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(57) Abstract

The present invention provides specific genetic deletions that result in an avirulent phenotype of a mycobacterium. These deletions may be used as phenotypic markers of providing a means for distinguishing between disease-producing and non-disease producing mycobacteria.

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VIRULENCE-ATTENUATING GENETIC DELETIONS

BACKGROUND OF THE INVENTION

Mycobacterium tuberculosis (MTB) infects over ten million people each year and kills over three million, making it the infectious agent causing the greatest mortality worldwide. In an effort to combat *Mycobacterium tuberculosis*, vaccination programs using a viable attenuated strain of *Mycobacterium bovis* called bacille Calmette-Guérin (BCG) have been established in more than 120 countries over the course of the last 5 decades. Although widely used and considered safe enough to administer to infants, the BCG vaccine is controversial for two principle reasons: 1) Efficacy for BCG vaccines against tuberculosis has varied from 0-85% in different clinical trials; and 2) Immunization with BCG sensitizes vaccinees to the tubercular antigens used in the tuberculin skin test, confounding attempts to discriminate between BCG immunization and TB infection. For these two reasons, especially the latter, BCG is not used in the United States where surveillance with the tuberculin test is preferred.

The original Pasteur BCG strain was developed by multiple (230 times) serial passages in liquid culture. BCG has never been shown to revert to virulence in animals indicating that the attenuating mutations in BCG are stable deletions and/or multiple mutations which cannot revert. However, the mutations which arose during serial passage of the original BCG strain have never been identified. Moreover, recent efforts to genetically complement BCG virulence with genomic libraries of virulent tubercle bacilli have also been unsuccessful again suggesting that multiple unlinked mutations are responsible for the attenuation of BCG virulence. The antigenicity of BCG and the characteristics leading to its avirulence are thus poorly understood.

SUMMARY OF THE INVENTION

The present invention provides specific genetic deletions that account for the avirulent phenotype of the bacille Calmette-Guérin (BCG) strain of *Mycobacterium bovis*. These deletions may be used as phenotypic markers of providing a means for distinguishing between disease-producing and non-disease producing mycobacteria.

In a preferred embodiment, this invention provides for nucleic acid sequences that are markers for avirulent or virulent mycobacteria. The sequences uniquely characterize the presence or absence of deletions that result in an avirulent phenotype. More specifically the sequence are either deletion junction sequence or deletion sequences or subsequences within deletion junction sequences or deletion sequences. Thus, this invention provides for a marker for an avirulent mycobacterium comprising a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of the second nucleic acid where the second nucleic acid or its complement includes BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3. In a particularly preferred embodiment, the marker specifically hybridizes under stringent conditions to a nucleic acid from BCG, but not to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or alternatively, the marker specifically hybridizes under stringent conditions to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, but not to a nucleic acid from BCG. The marker may be the full length BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3 or a subsequence within any of these regions. The marker may also include a nucleic acid having at least 80%, preferably 90%, more preferably 95%, and most preferably 98% percent sequence identity with BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, or BCGΔ3. The marker may also include a sequence selected from an open reading frame of a the deletion sequences BCGΔ1, BCGΔ2, BCGΔ3. Suitable open reading frames are indicated in Figures **4**, **5**, and **6.**

The above described marker may be a probe. The probe may be labeled by a number of means including, but not limited to radioactive, fluorescent, enzymatic, and colorimetric labels.

In another embodiment, this invention provides for polypeptides encoded by a subsequence of the BCGΔ1, BCGΔ2, or BCGΔ3 deletions. In particular, the subsequence may be selected from an open reading frame (ORF) present in one of these deletion sequences. This invention also provides for monoclonal or polyclonal antibodies that

specifically bind polypeptides encoded by one or more subsequences of the BCGΔ1, BCGΔ2, or BCGΔ3 deletions.

In still another embodiment, this invention provides for a recombinant cell comprising a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of the second nucleic acid where the second nucleic acid or its complement is BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, or BCGΔ3. The recombinant cell may be a mycobacterium. The recombinant cell may express a polypeptide encoded by any of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3. More preferably, the recombinant cell expresses a polypeptide encoded by an intact open reading frame present in any of these regions. The cell may also be a mycobacterium having one or more deletions in the BCGΔ1, BCGΔ2, or BCGΔ3 genomic regions where the deletions result in the attenuation of an otherwise virulent strain of mycobacterium and wherein the deletions are present in up to two of the genomic regions.

In still yet another embodiment, this invention provides a method of distinguishing between an attenuated and a virulent mycobacterium. The method involves detecting the presence or absence of a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of the second nucleic acid where the second nucleic acid or its complement is BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, or BCGΔ3. The first nucleic acid may include any of the markers described above. A particularly preferred marker is one that specifically hybridizes under stringent conditions to a nucleic acid from BCG, but not to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or alternatively, that specifically hybridizes under stringent conditions to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, but not to a nucleic acid from BCG. The method may involve amplifying either the first nucleic acid by any of a number of methods including, for example, polymerase chain reaction. The detection may involve detecting the first nucleic acid, for example, as in a Southern blot, or alternatively, detecting a polypeptide encoded by the first nucleic acid. More specifically, the polypeptide may be

a encoded by an open reading frame (ORF) selected from BCGΔ1, BCGΔ2, or BCGΔ3. The polypeptide may be visualized by a number of means well known to those of skill in the art including antibody hybridization such as direct or indirect binding of labeled antibody.

This invention additionally provides a method for determining whether an attenuated or a virulent Mycobacterium is present in a sample. This method involves providing a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of the second nucleic acid where the second nucleic acid or its complement is BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, or BCGΔ3; and hybridizing the first nucleic acid to the biological sample. The first nucleic acid may include any of the markers described above. A particularly preferred marker is one that specifically hybridizes under stringent conditions to a nucleic acid from BCG, but not to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or alternatively, that specifically hybridizes under stringent conditions to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, but not to a nucleic acid from BCG. The method may involve amplifying either the first nucleic acid by any of a number of methods including, for example, polymerase chain reaction. The detection may involve detecting the first nucleic acid, for example, as in a Southern blot, or alternatively, detecting a polypeptide encoded by the first nucleic acid. More specifically, the polypeptide may be a encoded by an open reading frame (ORF) selected from BCGΔ1, BCGΔ2, or BCGΔ3. The method may also include detecting the hybridized first nucleic acid. This may involve direct detection of a label or additionally involve an amplification step and subsequent detection of the amplified product.

Finally, this invention provides a method of producing an attenuated-virulence mycobacterium. This method involves deleting from the genomic DNA of a virulent mycobacterium a first nucleic acid that specifically hybridizes under stringent conditions with a second nucleic acid or a complement of said second nucleic acid where said second nucleic acid or complement of said second nucleic acid is selected from the group consisting of BCGΔ1, BCGΔ2, and BCGΔ3. The first nucleic acid may be BCGΔ1, BCGΔ2, or BCGΔ3, or alternatively, it may be a promoter, other control element or an open reading frame from BCGΔ1, BCGΔ2, or BCGΔ3.

Definitions

Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

The phrase "specifically detect" as used herein refers to the process of determining that a particular subsequence is present in a DNA sample. A DNA sequence may be specifically detected through a number of means known to those of skill in the art. These would include, but are not limited to amplification of the particular target sequence through polymerase chain reaction or ligase chain reaction, hybridization of the sequence to a labeled probe, and binding by labelled ligands or monoclonal antibodies. For a discussion of various means of detection of specific nucleic acid sequences see Perbal, B. *A Practical Guide to Molecular Cloning*, 2nd Ed. John Wiley & Sons, N.Y. (1988) which is incorporated herein by reference.

The phrase "select subsequence" is used herein to refer to a particular DNA subsequence that is of interest. It is often a predetermined or known sequence of nucleic acid bases. A select subsequence is typically chosen because of a unique sequence identity. Typically a select subsequence is targeted for DNA amplification and often is useful as a specific marker for the presence of a particular gene or a deletion of a particular nucleic acid sequence.

The term "oligonucleotide" refers to a molecule comprised of two or more deoxyribonucleotides or ribonucleotides. Oligonucleotides may include, but are not limited to, primers, probes, nucleic acid fragments to be detected, and nucleic acid controls. Oligonucleotides include naturally occurring nucleotides, chemically modified naturally occurring nucleotides and synthetic nucleotides. The exact size of an oligonucleotide depends on many factors and the ultimate function or use of the oligonucleotide.

The term "primer" refers to an oligonucleotide, whether natural or synthetic, capable of acting as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced, *i.e.*, in the presence of four different nucleoside triphosphates and an agent for polymerization (*i.e.*, DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. A primer is preferably a single-stranded oligodeoxyribonucleotide.

The appropriate length of a primer depends on the intended use of the primer but typically ranges from 15 to 25 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template.

The phrase "PCR primers competent to amplify" as used herein refers to a pair of PCR primers whose sequences are complementary to DNA subsequences immediately flanking the DNA subsequence (target sequence) which it is desired to amplify. The primers are chosen to bind specifically those particular flanking subsequences and no other sequences present in the sample. The PCR primers are thus preferably chosen to amplify the unique target sequence and no other. Alternatively, the PCR primers may be selected to bind to sequences other than the target sequence where the amplification products can be subsequently distinguished (*e.g.* where the desired amplified sequence is different in size than other amplified sequences).

"Amplifying" or "amplification", which typically refer to an "exponential" increase in target nucleic acid, are used herein to describe both linear and exponential increases in the number of a select target sequence of nucleic acid.

The term "antisense orientation" refers to the orientation of nucleic acid sequence from a structural gene that is inserted in an expression cassette in an inverted manner with respect to its naturally occurring orientation. When the sequence is double stranded, the strand that is the template strand in the naturally occurring orientation becomes the coding strand, and vice versa.

The term "deletion" refers to a region of a nucleic acid which is not present in an organism, but which is present in another related organism. In the context of mycobacteria, a deletion refers, *e.g.*, to a region of nucleic acid which is not present in one strain of mycobacteria, but which is present in another related strain. For instance, an avirulent mycobacterial strain can have a deletion in its genome relative to the genome of a related virulent mycobacterial strain.

The term "deletion junction" refers to the region of a nucleic acid spanning the insertion point of a deletion. Thus, where a region of a nucleic acid sequence is deleted (*i.e.* a deletion is present), the deletion junction spans the nucleotides that are immediately adjacent to the deletion. Conversely, where a region of a nucleic acid sequence is not

deleted (*i.e.* the deletion is absent), two deletion junctions are present, each spanning respectively one end of the deletion sequence and its flanking sequence.

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, such as a polynucleotide sequence of Figures 1, 2, or 3, or may comprise a complete cDNA or gene sequence.

Generally, a reference sequence is at least 10 nucleotides in length, frequently at least 20 to 25 nucleotides in length, and often at least 50 nucleotides in length. Sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a segment of at least 10 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 10 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences.

Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48: 443 (1970); by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. (USA)* 85: 2444 (1988), or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (*i.e.*, resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected.

The term "sequence identity" means that two polynucleotide sequences are identical (*i.e.*, on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned

sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.*, A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The term "identical" in the context of two nucleic acid or polypeptide sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence.

The terms "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. The isolated nucleic acid probes of this invention do not contain materials normally associated with their *in situ* environment, in particular nuclear, cytosolic or membrane associated proteins or nucleic acids other than those nucleic acids intended to comprise the nucleic acid probe itself.

The term "marker" refers to a characteristic which distinguishes one class of cells or compositions from a second class of cells or compositions. For instance, the deletions and deletion junctions described herein can be used to distinguish between strains (*e.g.*, virulent and avirulent strains) of mycobacteria. While markers are indicators of associated features or properties, as used herein, markers may also be used for purposes other than indicating the associated feature or property. Thus, for example, a nucleic acid marker of virulence identifies a particular nucleic acid which may be used in a variety of contexts other than simply indicating virulence.

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompassing known analogues of natural nucleotides that can function in a similar manner as naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence includes the complementary sequence thereof.

The term "operably linked" refers to functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates transcription of RNA corresponding to the second sequence.

The term "peptide" or "polypeptide" refers to an amino acid polymer which is encoded by a nucleic acid. The peptide or polypeptide may include naturally occurring or modified amino acids.

The terms "probe" or "nucleic acid probe" refer to a molecule that binds to a specific sequence or subsequence of a nucleic acid. A probe is preferably a nucleic acid which binds through complementary base pairing to the full sequence or to a subsequence of a target nucleic acid. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarily with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labelled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labelled such with, *e.g.*, biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the selected sequence or subsequence.

The term "labeled nucleic acid probe" refers to a nucleic acid probe that is bound, either covalently, through a linker, or through ionic, van der Waals or hydrogen "bonds" to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe.

The term "recombinant" when used with reference to a cell indicates that the cell replicates or expresses a nucleic acid, or expresses a peptide or protein encoded by DNA whose origin is exogenous to the cell. Recombinant cells can express genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also express genes found in the native form of the cell wherein the genes are re-introduced into the cell by artificial means.

The term "sample" refers to a material with which bacteria may be associated. Frequently the sample will be a "clinical sample" which is a sample derived from a patient. Such samples include, but are not limited to, sputum, blood, blood cells (*e.g.*, white cells), tissue or fine needle biopsy samples, urine, peritoneal fluid, and pleural fluid, or cells therefrom. It will be recognized that the term "sample" also includes supernatant from eukaryotic cell cultures (which may contain free bacteria), cells from cell or tissue culture, and other media in which it may be desirable to detect mycobacteria (*e.g.*, food and water).

The term "subsequence" in the context of a particular nucleic acid sequence refers to a region of the nucleic acid equal to or smaller than the specified nucleic acid.

The term "substantial identity" or "substantial similarity" indicates that a nucleic acid or polypeptide comprises a sequence that has at least 90% sequence identity to a reference sequence, or preferably 95%, or more preferably 98% sequence identity to the

reference sequence, over a comparison window of at least about 10 to about 100 nucleotides or amino acid residues. An indication that two polypeptide sequences are substantially identical is that one protein is immunologically reactive with antibodies raised against the second protein. An indication that two nucleic acid sequences are substantially identical is that the polypeptides which the first nucleic acids encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence-dependent and will be different with different environmental parameters. Generally, stringent conditions are selected to be about 5°C to 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0.2 molar at pH 7 and the temperature is at least about 60°C.

The term "uninterrupted reading frame" or "open reading frame" refers to a DNA sequence (*e.g.*, cDNA) lacking a stop codon or other intervening, untranslated sequence. An intact open reading frame refers to a full length uninterrupted reading frame or minor variations thereof.

The term "virulent" in the context of mycobacteria refers to a bacterium or strain of bacteria that replicates within a host cell or animal at a rate that is detrimental to the cell or animal within its host range. More particularly virulent mycobacteria persist longer in a host than avirulent mycobacteria. Virulent mycobacteria are typically disease producing and infection leads to various disease states including fulminant disease in the lung, disseminated systemic milliary tuberculosis, tuberculosis meningitis, and tuberculosis abscesses of various tissues. Infection by virulent mycobacteria often results in death of the host organism. Typically, infection of guinea pigs is used as an assay for mycobacterial virulence. In contrast, the term "avirulent" refers to a bacterium or strain of bacteria that either does not replicate within a host cell or animal within its host range, or replicates at a rate that is not significantly detrimental to the cell or animal.

The term BCG-like avirulence, as used herein refers to an attenuated virulence brought about by one of the deletions of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the complete sequence listing of the BCG deletion region 1 including flanking sequences. The deletion, designated BCGΔ1, is located between nucleotide 2327 and nucleotide 11126.

Figure 2 shows the complete sequence listing of the BCG deletion region 2 including flanking sequences. The deletion, designated BCGΔ2, is located between nucleotide 3382 and nucleotide 14071.

Figure 3 shows the complete sequence listing of the BCG deletion region 3 including flanking sequences. The deletion, designated BCGΔ3, is located between nucleotide 1406 and nucleotide 10673. "N" represents "A", "C", "G", or "T".

Figure 4 shows a map of the deletion sequence BCGΔ1. This map identifies the various open reading frames (ORFs) and indicates their location within the deletion sequence. Ribozome binding sites and homologies to the predicted encoded proteins are shown.

Figure 5 shows a map of the deletion sequence BCGΔ2. This map identifies the various open reading frames (ORFs) and indicates their location within the deletion sequence. Ribozomal binding sites and homologies to the predicted encoded proteins are shown.

Figure 6 shows a map of the deletion sequence BCGΔ3. This map identifies the various open reading frames (ORFs) and indicates their location within the deletion sequence. Ribozome binding sites and homologies to the predicted encoded proteins are shown. The sequence of a small region, estimated to be much less than 200 bp and located close to 9400 bp in Figure 3, remains to be determined. Therefore, the base pair coordinates given in the region 3 map 3' to the 9kb marker are approximations. The precise sequence determination of this region is likely to effect the length of open reading frames 3H and 3L.

Figure 7 illustrates the deletion junction regions of BCGΔ1, BCGΔ2, and BCGΔ3. The "terminal" deletion junction regions formed by the flanking sequences and the terminal regions of the deletion sequences are identified as BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, and BCGΔ3a, and BCGΔ3b. When the deletion is present (the deletion sequences

are missing) the respective "a" and "b" sequences will be juxtaposed, thereby forming deletion "spanning" junction sequences designated BCGΔ1ab, BCGΔ2ab, and BCGΔ3ab, respectively.

Figure 8 shows EcoRI and BamHI restricted chromosomal DNAs from *Mycobacterium bovis*, BCG Connaught, and *Mycobacterium tuberculosis* strains H37Ra, H37Rv, and Erdman probed with ^{32}P labeled BCG subtracted probe.

DETAILED DESCRIPTION

This invention reflects the discovery of genetic deletions in mycobacteria that result in an avirulent genotype such as is exhibited by the bacille Calmette-Guérin (BCG) mycobacterium. The original Pasteur bacille Calmette-Guérin (BCG) strain was developed by multiple (230 times) serial passages in liquid culture. BCG has never been shown to revert to virulence in animals indicating that the attenuating mutations in BCG are stable deletions and/or multiple mutations that cannot revert. The mutations that arose during serial passage of the original BCG strain were not previously known. Recent efforts to genetically complement BCG virulence with genomic libraries of virulent tubercle bacilli were unsuccessful, again suggesting that multiple unlinked mutations are responsible for the attenuation of BCG virulence.

The genetic deletions leading to the avirulent phenotype of BCG were identified by genomic subtractions between Connaught strain of BCG and MBV/MTB. The subtracted probe resulting from the genomic subtraction between BCG and the H37 Rv strain of *M. tuberculosis* was subsequently used to identify and clone three regions from a cosmid library of *Mycobacterium bovis* genomic DNA. Southern blot mapping and DNA sequence comparisons between BCG and *M. bovis* showed that three regions, designated regions 1-3, contained DNA segments of approximately 9 kb, 11 kb and 9 kb respectively, which are deleted in the Connaught strain of BCG. Precise deletion junctions were identified for each region by comparisons of BCG and corresponding virulent MBV sequences. The respective deletions, designated BCGΔ1, BCGΔ2 and BCGΔ3 are illustrated in Figures 1-3.

One of skill in the art will appreciate that the deletions encompassed by BCGΔ1, BCGΔ2 and BCGΔ3 may be utilized in a variety of contexts. For example, the deletions may be utilized to distinguish between avirulent and virulent strains of

mycobacteria thereby providing early detection of patients at risk for tuberculosis. This is of particular importance where mycobacteria are identified in a sample from a patient that has been previously vaccinated with BCG. In this context it may be critical to determine whether mycobacteria identified in a biological sample from such a patient are pathogenic.

In another embodiment, the preparation of mycobacteria containing the deletions of the present invention may provide superior vaccines to BCG which has long been known to have marginal efficacy. Thus, for example, a *Mycobacterium tuberculosis* may contain a full BCGΔ1 deletion or a smaller deletion within BCGΔ1 (*e.g.* one or more open reading frames) rendering it avirulent. An avirulent MTB will provide a more efficient vaccine because it is antigenically more similar to MTB than is BCG. Moreover, an MTB rendered avirulent by the production of smaller deletions within the deletion regions identified in this invention will present more antigenic determinants.

Since the loss of virulence is due to the loss of gene products expressed by the nucleic acid sequences comprising the deletion regions, the BCGΔ1, BCGΔ2 and BCGΔ3 deletion sequences and proteins encoded within these deletion sequences provide suitable targets for drug screening. Thus, the use of deleted sequences as targets to screen for drugs that inhibit or interfere with transcription, translation, or post-translational processing of proteins encoded by the deletion sequences, or with the deletion encoded polypeptides themselves, provides an assay for anti-mycobacterial agents. In particular, the use of reporter genes such as firefly luciferase (FFlux), ß-galactosidase (BGal), and the like, under the control of promoters present in the deletion sequence provide a rapid assay for drugs regulating activity originating in this region. Conversely, since the protein products of the deletion sequences are presumably expressed in virulent mycobacterial species, proteins expressed by deletion sequences may make good antigens for antimycobacterial vaccines.

Finally, as the viability of BCG demonstrates, deletion regions BCGΔ1, BCGΔ2 and BCGΔ3 are not required for mycobacterial growth and reproduction. Thus, these deletion regions provide good insertion points for the expression of heterologous DNA. The heterologous DNA sequences may be under the control of endogenous inducible or constitutive promoters typically found in the deletion sequences, or alternatively, they may be under the control of introduced promoters, either constitutive or inducible, exogenous to mycobacteria.

I. Detection of Deletions

As indicated above, the deletions identified in the present invention provide useful markers for the identification of an avirulent (or conversely a virulent) mycobacterial phenotype. Specifically, determination of avirulence simply requires the detection of the presence or absence of the deletion (either BCGΔ1, BCGΔ2, or BCGΔ3, or deletions within these regions). Where the deletion is present in the bacterial DNA, the bacterium expresses a BCG-like avirulent phenotype. Conversely, where the deletion is absent in the bacterial DNA, the bacterium does not express a BCG-like avirulence. While this may indicate that the bacterium is virulent, one of skill will appreciate that the bacterium may still be avirulent due to the presence of other mutations or deletions. Nevertheless, screening for the presence of the deletion provides a means of detecting a BCG-like avirulent mycobacterium.

Means of detecting deletions are well known to those of skill in the art. Generally, the deletions may be detected either by detecting the presence or absence of deletion junctions, or, alternatively, by detecting the presence or absence of the sequences contained within the deletion (deletion sequences). Where a nucleic acid sequence is deleted (*i.e.*, a deletion is present), the sequences that previously flanked the deleted sequence are juxtaposed, thereby forming a new deletion junction that spans the deletion. Detection of the presence of such a "spanning" deletion junction indicates the presence of the deletion and thus the avirulent phenotype.

Conversely, where the nucleic acid sequence is not deleted (the deletion is not present) the spanning junction sequence will be absent (See, *e.g.* Figure 7). The "terminal" deletion junction sequences flanking each endpoint of the deletion region are present and detection of these terminal deletion junctions indicates the absence of a deletion. Spanning deletion junction regions and terminal deletion junctions suitable for detecting the deletions of the present invention are illustrated in Figure 7 and in Table 1.

Table 1. Nucleic acid sequences comprising deletion junctions. The symbol "¦" indicates the insertion point of the deletion sequence. Deletion sequence bases are represented in lower case letters.

Junction	Nucleotide Sequence	Seq. ID
BCGΔ1a	CTGGTCGACGATTGGCACAT¦gcagccgtgggtgccgccgg	1

BCGΔ1b	gtgtcttcatcggcttccac¦CCAGCCGCCCGGATCCAGCA	2
BCGΔ2a	CAACTCCACGGCGACCACCC¦gcgcccccgctcgcactaga	3
BCGΔ2b	gcccacccggtcgagcaccc¦CGATGATCTTCTGTTTGACC	4
BCGΔ3a	CACCTCGACCACGGCCAACC¦gtggacctgtgagatacact	5
BCGΔ3b	tcagcagtccacggccaacc¦CCGCACCAACACCTTCCACC	6
BCGΔ1ab	CTGGTCGACGATTGGCACAT¦CCAGCCGCCCGGATCCAGCA	7
BCGΔ2ab	CAACTCCACGGCGACCACCC¦CGATGATCTTCTGTTTGACC	8
BCGΔ3ab	CACCTCGACCACGGCCAACC¦CCGCACCAACACCTTCCACC	9

Where a deletion is detected by determining the presence or absence of sequences contained within the deletion (deletion sequences), the absence of deletion sequences indicates the presence of a deletion and thus an avirulent phenotype. Conversely, the presence of deletion sequences indicates the absence of a deletion. Deletion sequences that provide suitable targets for detecting the deletions of the present invention are provided in Figures 1, 2 and 3.

A) Isolation of DNA for Detection of Mycobacterium Genomic Deletions

In a preferred embodiment, DNA is obtained from mycobacteria. As used herein, the term "mycobacteria" refers to any bacteria of the family *Mycobacteriaceae* (order *Actinomycetales*) and includes, but is not limited to, *Mycobacterium tuberculosis*, *Mycobacterium avium complex*, *Mycobacterium kansasii*, *Mycobacterium scrofulaceum*, *Mycobacterium bovis and Mycobacterium leprae*. These species and groups and others are described in Baron, S., ed. *Medical Microbiology*, 3rd Ed. (1991) Churchill Livingstone, New York, which is incorporated herein by reference.

The identification of deletions using a DNA marker requires that the DNA sequence be accessible to the particular probes used or to the components of the amplification system if the DNA sequence is to be amplified. In general, this accessibility is ensured by isolating the nucleic acids from the sample.

A variety of techniques for extracting nucleic acids from biological samples are known in the art. For example, see those described by Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New

York, (1985), by Han, *et al. Biochemistry*, 26: 1617-1625 (1987) and by Du, *et al. Bio/Technology*, 10: 176-181 (1992), which are incorporated herein by reference.

Alternatively, if the sample is readily disruptable, the nucleic acid need not be purified prior to amplification by the PCR technique, *i.e.*, if the sample is comprised of cells, particularly peripheral blood lymphocytes or monocytes, lysis and dispersion of the intracellular components may be accomplished merely by suspending the cells in hypotonic buffer or boiling them in a low concentration of alkali (*i.e.* 10 mM NaOH).

In a preferred embodiment, DNA is extracted from mycobacteria as described in Example 1.

B) Detection of Deletions Using Hybridization Probes

In one embodiment the avirulence deletions are detected by contacting DNA obtained from the mycobacterium with a probe that specifically binds an entire deletion junction region or a subsequence of that region and does not specifically bind to any other DNA sequences in the sample. Alternatively, a probe that specifically binds the entire deleted region or subsequence of that region and does not specifically bind to any other sequences in the sample is also suitable. While such probes may be proteins, oligonucleotide probes are preferred. Typically, the sequence of the oligonucleotide probe is chosen to be complementary to a select subsequence unique to the deletion junction or the deletion sequence, whose presence or absence is to be detected. Under stringent conditions the probe will hybridize with the select subsequence forming a stable duplex.

The probe is typically labeled. Detection of the label in association with the target DNA indicates either the presence or absence of the deletion. The probe may be used to detect the deletion junction or deletion sequences directly in a DNA sample without amplification of the deletion subsequences. In one embodiment, unamplified DNA sequences are probed using a Southern blot. The DNA of the sample is immobilized, on a solid substrate, typically a nitrocellulose filter or a nylon membrane. The substrate-bound DNA is then hybridized with the labeled probe under stringent conditions and non-specifically hybridized probe is washed away. Labeled probe detected in association with the immobilized mycobacterial sequences (*e.g.* bound to the substrate) indicates the presence of deletion sequences (*e.g.* BCGΔ1, BCGΔ2, or BCGΔ3) and therefore the absence of the deletion. Means for detecting specific DNA sequences are well known to those of skill in

the art. Protocols for Southern blots as well as other detection methods are provided in Maniatis, *et al. Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY (1982), which is incorporated herein by reference.

In another embodiment, the mycobacterial DNA subsequences are themselves labeled. They are then hybridized, under stringent conditions, with a probe immobilized on a solid substrate. Detection of the label in association with the immobilized probe indicates the presence or absence of the deletion.

In a preferred embodiment, the deletion junction sequences or subsequences or the deletion sequences or subsequences may be amplified by a variety of DNA amplification techniques (for example via cloning, polymerase chain reaction, ligase chain reaction, transcription amplification, *etc.*) prior to detection using a probe. Because the copy number of mycobacterial sequences bearing the virulence-attenuating deletions is low, the use of unamplified mycobacterial DNA results in an assay of low sensitivity. Amplification of mycobacterial DNA increases sensitivity of the assay by providing more copies of possible target subsequences. In addition, by using labeled primers in the amplification process, the mycobacterial DNA sequences are labeled as they are amplified.

C) Selection of Probes for Detection of the Deletion Junction Sequences or the Deletion Sequences

Full length sequences are provided for the deletions BCGΔ1, BCGΔ2, and BCGΔ3 in Figures 1, 2 and 3 respectively. Using these sequence listings, one of skill in the art may easily determine appropriate probes or primers for the detection of the presence or absence of the deletion junctions or the deletion sequences. Generally speaking, a probe will be selected that hybridizes to the target junction sequences or deletion sequences, but not to other mycobacterial nucleic acid sequences under stringent conditions. The design of hybridization probes is well known in the art. See, for example, Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989), which is incorporated herein by reference.

In a preferred embodiment, the probe is an oligonucleotide sequence complementary to a subsequence comprising a deletion junction (*e.g.* BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, and BCGΔ3ab) or a

sequence complementary to a subsequence of a deletion sequence (*e.g.* BCGΔ1, BCGΔ2, and BCGΔ3). The probe preferably has destabilizing mismatches with subsequences from other regions of the mycobacterial genome.

The exact length of the probe depends on many factors including the length of conserved regions around the deletions, the degree of sequence specificity desired, and the amount of internal complementarity within the probe. Such probes are preferably 17 to 25 bases in length. One of skill will recognize that longer probes specifically hybridize at higher temperatures. Generally, stringent conditions are selected to be about 5°C to 20°C, more preferably about 10°C, lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. Under stringent conditions, the probe will specifically hybridize to a nucleic acid sequence from an avirulent mycobacterium such as BCG, but not to a nucleic acid sequence from a virulent mycobacterium such as MTB or MBV. Alternatively, Under stringent conditions, the probe will specifically hybridize to a nucleic acid sequence from a avirulent mycobacterium such as MTB or MBV, but not to a nucleic acid sequence from an avirulent mycobacterium such as BCG.

Oligonucleotide probes can be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences and direct chemical synthesis by a method such as the phosphotriester method of Narang *et al. Meth. Enzymol*, 68: 90-99 (1979); the phosphodiester method of Brown *et al.*, *Meth. Enzymol.* 68:109-151 (1979); the diethylphosphoramidite method of Beaucage *et al.*, *Tetra. Lett.*, 22: 1859-1862 (1981); and the solid support method of U.S. Patent No. 4,458,066.

Probe detectability may be increased by the attachment of a label. As used herein, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads™), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*) beads.

Methods for attaching labels to probes, primers, and antibodies are well known to those of skill in the art. For example, the probe can be labeled at the 5'-end with ^{32}P by incubating the probe with ^{32}P-ATP and polynucleotide kinase (see Perbal, *A*

Practical Guide to Molecular Cloning, 2nd ed. John Wiley, N.Y. (1988)). Other labels may be joined to the probe directly or through linkers. They may be located at the ends of the probe or internally. Methods of attaching labels may be found in Connell, *et al.*, *Bio/Techniques* 5: 342 (1987), U.S. Patent Nos. 4,914,210, 4,391,904 and 4,962,029, which are incorporated herein by reference. In addition, kits for labelling oligonucleotides are widely available. See, for example, Boehringer Mannheim Biochemicals (Indianapolis, IN) for "Genius" labeling kits based on dioxigenin technology and Clonetech (South San Francisco, CA) for a variety of direct and indirect oligonucleotide labeling reagents.

D) Detection of Deletions Conferring Avirulence Through Amplification of Unique Subsequences

Deletions are particularly amenable to detection without the use of a hybridization probe. In a preferred embodiment, subsequences are amplified that include a deletion junction. The amplified deletion junction may be a "spanning" deletion junction in which case where the deletion is present (*i.e.* the deletion sequences are absent), the amplification product is a specific DNA incorporating the deletion junction sequence spanning the deletion (*e.g.* incorporating flanking sequences from both sides of the deleted sequence). Where the deletion is absent (*i.e.* deletion sequences are present) and primers are selected so that there are no priming sites within the deletion sequences, amplification is non-existent or alternatively provides a complex mixture of non-specifically amplified fragments. Alternatively, amplification primers may be selected that specifically hybridize to deletion sequences, as long as they are selected to amplify sequences that are distinguishable from the sequence amplified when the deletion is present.

Alternatively, the amplification product may be subsequence of a "terminal" deletion junction in which case absence of the deletion (*i.e.* the deletion sequences are present) will result in the amplification of the specifically targeted nucleic acid. Conversely, where the deletions are present (*i.e.* the deletion sequences are absent) there will be no specific amplification of a terminal deletion junction.

Amplification products may be separated by size for characterization. Size separation may be accomplished by a variety of means known to those of skill in the art.

These methods include, but are not limited to electrophoresis, density gradient centrifugation, liquid chromatography, and capillary electrophoresis. In a preferred embodiment, the fragments are separated by agarose gel electrophoresis. The bands are then stained with a marker to visualize them such as ethidium bromide and the gel is visualized, *e.g.*, using ultraviolet light.

As described above, an agarose gel typically shows 1 band if the deletion is present, reflecting amplification of the deletion-spanning sequence. Where the deletion is absent, amplification results in either no bands, where there are no sequences within the deletion to which the amplification primers may hybridize, or a smear where there is non-specific amplification, or a series of discrete bands distinguishable from the band representing the deletion-spanning sequence where primers are chosen that hybridize to deletion sequences.

E) Selection of Primers for Amplification of Avirulence Deletions

Amplification of deletion junction sequences or subsequences or deletion sequences or subsequences may be accomplished by methods well known in the art, which include, but are not limited to polymerase chain reaction (PCR) (Innis, *et al.*, *PCR Protocols. A guide to Methods and Application.* Academic Press, Inc. San Diego, (1990), which is incorporated herein by reference), ligase chain reaction (LCR) (see Wu and Wallace, *Genomics*, 4: 560 (1989), Landegren, *et al.*, *Science*, 241: 1077 (1988) and Barringer, *et al.*, *Gene*, 89: 117 (1990), which are incorporated herein by reference), transcription amplification (see Kwoh, *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 86: 1173 (1989) which is incorporated herein by reference), and self-sustained sequence replication (see Guatelli, *et al.*, *Proc. Nat. Acad. Sci. (U.S.A.)*, 87: 1874 (1990) which is incorporated herein by reference), each of which provides sufficient amplification so that the target sequence can be detected by nucleic acid hybridization to a probe or by electrophoretic separation. Alternatively, methods that amplify the hybridization probe to detectable levels can be used, such as Qβ-replicase amplification. See, for example, Kramer, *et al. Nature*, 339: 401 (1989), Lizardi, *et al. Bio/Technology*, 6: 1197 (1988), and Lomell, *et al.*, *Clin. Chem.* 35: 1826 (1989) which are incorporated herein by reference.

In a preferred embodiment, amplification is by polymerase chain reaction using a pair of primers that flank and thereby amplify a selected deletion junction subsequence. Selection of primers is readily apparent to one of skill in the art using the sequence listings of the present invention. For example, a pair of PCR primers 5'-TCGACGATTGGCACAT-3' (T_m=55°C) and 5'-TCCCTCCCTGTATTTGTAT-3' (T_m=56°C) will amplify a 469 base pair sequence including the BCGΔ1a deletion junction, while 5'-CGTTCTTCGGAGGTTTC-3' (T_m=56°C) and 5'-GGCGGCTGGGTGGA-3' (T_m=60°C) will amplify a 471 base pair sequence including the BCGΔ1b deletion junction.

F) Detection of Deletions through Detection of Expression Products of Deletion Sequences

In addition to the detection of deletions by the detection of either the deletion junction sequences or the deletion sequences, one may detect the absence of the deletion by detecting the expression products of the deletion sequences. Thus, for example, where the deletion sequences express a protein, the presence of that protein indicates the absence of the deletion and thus is indicative of a virulent (non BCG-like) phenotype. Such proteins are referred to herein as "deletion polypeptides".

Means of determining proteins expressed by particular nucleic acid sequences are well known to those of skill in the art. Typically this involves determining the longest open reading frame. This may be aided by the identification of initiation sites (*e.g.* ribozome binding sites). The protein encoded by the largest open reading frame is determined using codon preferences for the specific organism from which the nucleic acid is obtained. The polypeptide sequence listing may then be compared against a sequence database, *e.g.* GenBank, to determine other sequences sharing substantial sequence identity with the calculated sequence. The expression of the protein may be verified by isolating and then sequencing proteins having the predicted length and charge characteristics.

Once deletion polypeptides are identified they may be detected by routine methods well known to those of skill in the art. Typically this involves isolating and then detecting the polypeptide. The polypeptide may be isolated by a number of means well known to those of skill in the art. This includes typical methods of protein

purification such as high performance liquid chromatography (HPLC), electrophoresis, capillary electrophoresis, hyperdiffusion chromatography, thin layer chromatography, and the like. Methods of purifying and detecting proteins are well known to those of skill in the art (see, *e.g.*, *Methods in Enzymology Vol. 182: Guide to Protein Purification*, M. Deutscher, ed. Vol. 182 (1990), which is incorporated herein by reference).

Alternatively, deletion polypeptides sequences may be detected using immunoassays utilizing antibodies specific for the deletion polypeptides. The production of such antibodies and their use in immunoassays is detailed below.

G) Antibodies to Deletion Polypeptides

Antibodies can be raised to the polypeptides encoded by the nucleic acids corresponding to the open reading frames present in the deletion regions of the present invention (deletion polypeptides). As used herein "antibodies" include immunoglobulin or a population of immunoglobins which specifically bind to an antigen. Thus an antibody may be monoclonal or polyclonal including individual, allelic, strain, or species variants, and fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms. Additionally, antibodies can be raised to these polypeptides in either their native configurations or in non-native configurations. Anti-idiotypic antibodies may also be used.

1) Antibody Production

A number of immunogens may be used to produce antibodies specifically reactive with deletion polypeptides. Recombinant polypeptides are the preferred immunogen for the production of monoclonal or polyclonal antibodies. Naturally occurring polypeptides may also be used either in pure or impure form. Synthetic peptides made using sequences described herein may also used as immunogens for the production of antibodies.

Recombinant polypeptides are expressed in eukaryotic or prokaryotic cells and purified using standard techniques. The polypeptide is injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies may be generated for subsequent use in immunoassays to measure the presence and quantity of the polypeptide.

Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen, preferably a purified deletion polypeptide is mixed with an adjuvant and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired. *See, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY, which are incorporated herein by reference.

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Description of techniques for preparing such monoclonal antibodies may be found in, *e.g.*, Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane (1988) *Antibodies: A Laboratory Manual* CSH Press; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and particularly in Kohler and Milstein (1975) *Nature* 256: 495-497, which discusses one method of generating monoclonal antibodies.

Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are then fused with myeloma cells (*See*, Kohler and Milstein (1976) *Eur. J. Immunol.* 6: 511-519, incorporated herein by reference). The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*.

Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells is enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse *et al.* (1989) *Science* 246: 1275-1281. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B

cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Other suitable techniques involve selection of libraries of antibodies in phage or similar vectors. See, Huse *et al. Science* 246: 1275-1281 (1989); and Ward, *et al. Nature* 341: 544-546 (1989). The polypeptides and antibodies of the present invention are used with or without modification, including chimeric antibodies. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents, teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced. See, Cabilly, U.S. Patent No. 4,816,567; and Queen *et al. Proc. Nat'l Acad. Sci. USA* 86: 10029-10033 (1989).

Antibodies, including binding fragments and single chain versions, against predetermined fragments of deletion polypeptides can be raised by immunization of animals with conjugates of the fragments with carrier proteins as described above. Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies can be screened for binding to normal or defective polypeptides, or screened for agonistic or antagonistic activity, *e.g.*, mediated through a receptor. These monoclonal antibodies will usually bind with at least a K_D of about 1 mM, more usually at least about 300 μM, and most preferably at least about 0.1 μM or better.

The antibodies of this invention can also be used for affinity chromatography in isolating deletion polypeptides. Columns can be prepared where the antibodies are linked to a solid support, *e.g.*, particles, such as agarose, Sephadex, or the like, where a bacterial lysate, or recombinant cell lysate is passed through the column, washed, and treated with increasing concentrations of a mild denaturant, whereby purified deletion polypeptides are released.

The antibodies can be used to screen expression libraries for particular expression products. Usually the antibodies in such a procedure will be labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

In a preferred embodiment, antibodies to deletion polypeptides are used for the identification of cell populations expressing the polypeptides. By assaying the expression products of cells expressing the polypeptides it is possible to diagnose bacterial infections.

Antibodies raised against each polypeptide are useful to raise anti-idiotypic antibodies. These will be useful in detecting or diagnosing various immunological conditions related to the presence of the respective antigens.

2) Immunoassays

A particular deletion polypeptide can be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures in general, see Stites and Terr (eds.) 1991 *Basic and Clinical Immunology* (7th ed.). Moreover, the immunoassays of the present invention can be performed in any of several configurations, *e.g.*, those reviewed in Maggio (ed.) (1980) *Enzyme Immunoassay* CRC Press, Boca Raton, Florida; Tijan (1985) "Practice and Theory of Enzyme Immunoassays," *Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers B.V., Amsterdam; and Harlow and Lane *Antibodies, A Laboratory Manual*, *supra*, each of which is incorporated herein by reference. See also Chan (ed.) (1987) *Immunoassay: A Practical Guide* Academic Press, Orlando, FL; Price and Newman (eds.) (1991) *Principles and Practice of Immunoassays* Stockton Press, NY; and Ngo (ed.) (1988) *Non-isotopic Immunoassays* Plenum Press, NY.

Immunoassays for measurement of deletion polypeptides can be performed by a variety of methods known to those skilled in the art. In brief, immunoassays to measure the protein can be, *e.g.*, competitive or noncompetitive binding assays. In competitive binding assays, the sample to be analyzed competes with a labeled analyte for specific binding sites on a capture agent bound to a solid surface. Preferably the capture agent is an antibody specifically reactive with a deletion polypeptide produced as described above. The concentration of labeled analyte bound to the capture agent is inversely proportional to the amount of free analyte present in the sample.

In a competitive binding immunoassay, the deletion polypeptide present in the sample competes with labelled protein for binding to a specific binding agent, for example, an antibody specifically reactive with a particular deletion polypeptide. The binding agent is, *e.g.*, bound to a solid surface to produce separation of bound labelled polypeptide from the unbound labelled polypeptide. Alternately, the competitive binding assay may be conducted in liquid phase and any of a variety of techniques known in the art may be used to separate the bound labelled protein from the unbound labelled protein. Following separation, the amount of bound labeled protein is determined. The amount of polypeptide present in the sample is inversely proportional to the amount of labelled polypeptide binding.

Alternatively, a homogenous immunoassay may be performed in which a separation step is not needed. In these immunoassays, the label on the protein is altered by the binding of the protein to its specific binding agent. This alteration in the labelled protein results in a decrease or increase in the signal emitted by label, so that measurement of the label at the end of the immunoassay allows for detection or quantitation of the polypeptide.

Deletion polypeptides may also be detected by a variety of noncompetitive immunoassay methods. For example, a two-site, solid phase sandwich immunoassay may be used. In this type of assay, a binding agent for the protein, for example an antibody, is attached to a solid support. A second protein binding agent, which is also an antibody, and which binds the protein at a different site, is labelled. After binding at both sites on the protein, the unbound labelled binding agent is removed and the labelled binding agent bound to the solid phase is measured. The amount of labelled binding agent bound is directly proportional to the amount of polypeptide in the sample.

Western blot analysis can be used to determine the presence of a deletion polypeptide in a sample. Electrophoresis is carried out, for example, on a bacterial sample suspected of containing the deletion polypeptide. Following electrophoresis to separate the proteins, and transfer of the proteins to a suitable solid support such as a nitrocellulose filter, the solid support is incubated with an antibody reactive with the protein. This antibody is labelled, or alternatively may be it is detected by subsequent incubation with a second labelled antibody that binds the primary antibody.

The immunoassay formats described above employ labelled assay components. The label can be in a variety of forms as described above. The choice of label depends on sensitivity required, ease of conjugation with the compound, stability requirements, and available instrumentation. For a review of various labelling or signal producing systems which may be used, see U.S. Patent No. 4,391,904, which is incorporated herein by reference.

Antibodies reactive with a particular protein can also be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures applicable to the measurement of antibodies by immunoassay techniques, see Stites and Terr (eds.) *Basic and Clinical Immunology* (7th ed.) *supra*; Maggio (ed.) *Enzyme Immunoassay*, *supra*; and Harlow and Lane *Antibodies, A Laboratory Manual*, *supra*.

In brief, immunoassays to measure antisera reactive with polypeptides include competitive and noncompetitive binding assays. In competitive binding assays, the sample analyte competes with a labeled analyte for specific binding sites on a capture agent bound to a solid surface. Preferably the capture agent is a purified recombinant deletion polypeptide as described above. Other sources of polypeptides, including isolated or partially purified naturally occurring protein, can also be used. Noncompetitive assays are typically sandwich assays, in which the sample analyte is bound between two analyte-specific binding reagents. One of the binding agents is used as a capture agent and is bound to a solid surface. The second binding agent is labelled and is used to measure or detect the resultant complex by visual or instrument means. A number of combinations of capture agent and labelled binding agent can be used. A variety of different immunoassay formats, separation techniques and labels can be also be used similar to those described above for the measurement of deletion polypeptides.

II. Preparation of Deletion-Containing Mycobacteria

Mycobacteria containing specific deletions may be prepared by using methods of homologous recombination well known to those of skill in the art. In brief, homologous recombination is a natural cellular process which results in the scission of two nucleic acid molecules having identical or substantially similar (*i.e.* "homologous") sequences, and the ligation of the two molecules such that one region of each initially

present molecule is now ligated to a region of the other initially present molecule (Sedivy, *Bio/Technol.*, 6: 1192-1196 (1988).

Homologous recombination is exploited by a number of various methods of "gene targeting" well known to those of skill in the art. (*see, for example*, Mansour *et al. Nature*, 336: 348-352 (1988); Capecchi *Trends Genet.* 5: 70-76 (1989); Capecchi *Science* 244: 1288-1292 (1989); Capecchi *et al.* pages 45-52 In: *Current Communications in Molecular Biology*, Capecchi, M.R. (ed.), Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989); Frohman *et al. Cell* 56: 145-147 (1989)). Some approaches focus on increasing the frequency of recombination between two DNA molecules by treating the introduced DNA with agents which stimulate recombination (*e.g.* trimethylpsoralen, UV light, *etc.*), however, most approaches utilize various combinations of selectable markers to facilitate isolation of the transformed cells.

One such selection method is termed positive/negative selection (PNS) (Thomas and Cappechi *Cell* 51: 503-512 (1987)). This method involves the use of two selectable markers: one a positive selection marker such as the bacterial gene for neomycin resistance (*neo*^{r}); the other a negative selection marker such as the herpes virus thymidine kinase (*tk*) gene. *Neo*^{r} confers resistance to the drug G-418, while herpes *tk* renders cells sensitive to the nucleoside analog gangcyclovir (GANC) or 1-(2-deoxy-2-fluoro-b-d-arabinofuranosyl)-5-iodouracil (FIAU). The DNA encoding the positive selection marker in the transgene (*e.g.* *neo*^{R}) is generally linked to an expression regulation sequence that allows for its independent transcription in mycobacteria. It is flanked by first and second sequence portions of at least a part of the deletion or deletion flanking sequences.

These first and second sequence portions target the transgene to a specific nucleotide sequence. A second independent expression unit capable of producing the expression product for a negative selection marker, *e.g.* for herpes virus tk is positioned adjacent to or in close proximity to the distal end of the first or second portions of the first DNA sequence. Upon transfection, some of the mycobacteria incorporate the transgene by random integration, others by homologous recombination between the endogenous allele and sequences in the transgene. As a result, one copy of the targeted nucleic acid is disrupted by homologous recombination with the-transgene with simultaneous loss of the sequence encoding herpes tk gene. Random integrants, which

occur via the ends of the transgene, contain herpes tk and remain sensitive to GANC or FIAU. Therefore, selection, either sequentially or simultaneously with G418 and GANC enriches for transfected mycobacteria containing the transgene integrated into the genome by homologous recombination.

Methods of homologous recombination in mycobacteria are described in greater detail by Ganjam *et al. Proc. Natl. Acad. Sci. USA*, 88: 5433-5437 (1991) and Aldovini *et al., J. Bacteriol.*, 175: 7282-7289 (1993) which are incorporated herein by reference.

III. Screening for Drug Susceptibility/Therapeutics

The expression products of the open reading frames in the BCGΔ1, BCGΔ2, and BCGΔ3 deletions of the present invention are targets for anti-mycobacterial drugs. To determine particularly suitable drug targets, open reading frames and surrounding expression control sequences are introduced into avirulent strains of mycobacteria, alone or in combination with other open reading frame regions to determine which regions are critical for virulence. Once particular genes are identified as critical for virulence, anti-mycobacterial agents are designed to inhibit expression of the critical genes, or to attack the critical gene products. For instance, antibodies are generated against the critical gene products and used as prophylactic or therapeutic agents. Alternatively, small molecules can be screened for the ability to selectively inhibit expression of the critical gene products, *e.g.*, using recombinant expression systems which include the gene's endogenous promoter. These small molecules are then used as therapeutics, or prophylactic agents to inhibit mycobacterial virulence.

In another embodiment, anti-mycobacterial agents which render a virulent mycobacterium avirulent can be operably linked to expression control sequences and used to transform a virulent mycobacterium. Such anti-mycobacterial agents inhibit the replication of a specified mycobacterium upon transcription or translation of the agent in the mycobacterium.

Such transformed mycobacteria are useful as vaccine components, and as components of immunological infectivity assays. For instance, an animal's blood can be monitored for the presence of anti-mycobacterial antibodies using the procedures described herein, using transformed avirulent mycobacterial components in various

immunological assays. Anti-mycobacterial agents useful in this invention include, without limitation, antisense genes, ribozymes, decoy genes, transdominant proteins and suicide genes.

An antisense nucleic acid is a nucleic acid that, upon expression, hybridizes to a particular mRNA molecule, to a transcriptional promoter or to the sense strand of a gene. By hybridizing, the antisense nucleic acid interferes with the transcription of a complementary DNA, the translation of an mRNA, or the function of a catalytic RNA. Antisense molecules useful in this invention include those that hybridize to gene transcripts in the region of the deletions of the invention, particularly deletion region 1.

A ribozyme is a catalytic RNA molecule that cleaves other RNA molecules having particular nucleic acid sequences. Ribozymes useful in this invention are those that cleave deletion gene transcripts. Examples include hairpin and hammerhead ribozymes.

A decoy nucleic acid is a nucleic acid having a sequence recognized by a regulatory DNA binding protein (*i.e.*, a transcription factor). Upon expression, the transcription factor binds to the decoy nucleic acid, rather than to its natural target in the genome. Useful decoy nucleic acid sequences include any sequence to which a transcription factor binds in the deletion regions of the present invention.

A transdominant protein is a protein whose phenotype, when supplied by transcomplementation, will overcome the effect of the native form of the protein. For instance, an avirulent mycobacterium can be rendered virulent by introducing transdominant proteins from deletion region 1.

A suicide gene produces a product which is cytotoxic. In the vectors of the present invention, a suicide gene is operably linked to an inducible expression control sequences which is stimulated upon infection of a cell by a mycobacterium.

IV. Use of Expressed "Deletion Proteins" in a Vaccine

The deletion polypeptides encoded by the open reading frames in BCGΔ1, BCGΔ2, and BCGΔ3 may be recombinantly expressed and used as components of immunological assays as described above or in vaccines. Expression of polypeptides

encoded by the open reading frames of the BCGΔ1, BCGΔ2, or BCGΔ3 deletions may be accomplished by means well known to those of skill in the art.

In brief, the expression of natural or synthetic nucleic acids encoding deletion polypeptides will typically be achieved by operably linking the DNA or cDNA to a promoter (which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of polynucleotide sequence encoding deletion polypeptides.

To obtain high level expression of a cloned gene, such as those polynucleotide sequences encoding deletion polypeptides, it is desirable to construct expression plasmids which contain, at the minimum, a promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. The expression vectors may also comprise generic expression cassettes containing at least one independent terminator sequence, sequences permitting replication of the plasmid in both eukaryotes and prokaryotes, *i.e.*, shuttle vectors, and selection markers for both prokaryotic and eukaryotic systems. For detailed techniques employed in the recombinant expression of deletion proteins *see*, for example, Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory (1989)), *Methods in Enzymology*, Vol. 152: *Guide to Molecular Cloning Techniques* (Berger and Kimmel (eds.), San Diego: Academic Press, Inc. (1987)), or *Current Protocols in Molecular Biology*, (Ausubel, *et al.* (eds.), Greene Publishing and Wiley-Interscience, New York (1987), all of which are incorporated herein by reference.

The expressed deletion polypeptides may be used in a variety of assays. For example, the deletion polypeptides can be used as reagents in immunoblot assays to test whether a patient was previously exposed to virulent mycobacteria (*i.e.*, to test whether the patient has antibodies to the deletion polypeptide). These assays have the advantage of discriminating between previous exposure to an avirulent mycobacterium (*e.g.*, one used in a vaccine) and exposure to a virulent mycobacterium. Thus, vaccinated individuals can be tested for antibodies to the virulent mycobacterium without regard to whether the patient has been vaccinated with an avirulent mycobacterium.

The deletion polypeptides can also be used as antigenic vaccine components to direct antibodies to elements which are critical for virulence. These polypeptides can be added to existing vaccines (*e.g.*, those based upon avirulent mycobacteria and which lack the deletion polypeptide) to supplement the range of antigenicity conferred by the vaccine, or they may be used apart from other mycobacterial antigens. The vaccines of the invention contain as an active ingredient an immunogenically effective amount of a deletion polypeptide or of a recombinant vector which includes the deletion polypeptide. The immune response can include the generation of antibodies; activation of cytotoxic T lymphocytes (CTL) against cells presenting peptides derived from the polypeptides or other mechanisms well known in the art. See *e.g.* Paul *Fundamental Immunology Third Edition* published by Raven press New York (incorporated herein by reference) for a description of immune response. Useful carriers are well known in the art, and include, for example, thyroglobulin, albumins such as human serum albumin, tetanus toxoid, and polyamino acids such as poly(D-lysine:D-glutamic acid). The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, and further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art.

The compositions are suitable for single administrations or a series of administrations. When given as a series, inoculations subsequent to the initial administration are given to boost the immune response and are typically referred to as booster inoculations.

The vaccine compositions of the invention are intended for parenteral, topical, oral or local administration. Preferably, the pharmaceutical compositions are administered parenterally, *e.g.*, intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration that comprise a solution of the agents described above dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile

solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient and more preferably at a concentration of 25%-75%.

For aerosol administration, the polypeptides are preferably supplied in finely divided form along with a surfactant and propellant. The surfactant should be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. A carrier can also be included, as desired, as with, *e.g.*, lecithin for intranasal delivery.

The amount of vaccine administered to the patient will vary depending upon the composition being administered, the physiological state of the patient and the manner of administration.

Live attenuated recombinant viruses which include the deletion polypeptide, such as recombinant vaccinia or adenovirus vectors, are convenient alternatives as vaccines because they are inexpensive to produce and are easily transported and administered. Vaccinia vectors and methods useful in immunization protocols are described, for example, in U.S. Patent No. 4,722,848, incorporated herein by reference.

Deletion sequences and subsequences of this invention may also be used in methods of genetic immunization. Briefly, genetic immunization involves transfecting

cells *in vivo* with nucleic acids encoding pathogen specific antigens. The transformed host cells then express the antigen thereby stimulating the host immune system.

In the present invention, antigen-encoding deletion region sequences are used to transform mammalian host cells thereby resulting in the expression of the antigen by the host. This provokes an immune response by the host against the expressed antigen thereby conferring immunity on the host. Methods of genetic immunization are well known to those of skill in the art (see, *e.g.*, Wang *et al. Proc. Natl. Acad. Sci. USA*, 90: 4156-4160 (1993); Ulmer *et al., Science*, 259: 1745-1749 (1993); Fynan *et al. DNA Cell Biol.*, 12: 785-789 (1993); Fynan *et al. Proc. Natl. Acad. Sci. USA*, 90: 11478-11482 (1993); Robinson *et al. Vaccine*, 11: 957-960 (1993); and Martinon *et al. Eur. J. Immunol.*, 23: 1719-1722 (1993), which are incorporated herein by reference.

VI. Use of Promoters within Deletion Sequences for Expression of Recombinant Proteins

Bacille Calmette-Guérin (BCG) contains all three deletions (BCGΔ1, BCGΔ2, and BCGΔ3) and yet is able to grow and reproduce indicating that the sequences contained within the deletion are not essential for bacterial viability. These deletion regions therefore make good target sites for the insertion of heterologous DNA as mycobacteria are tolerant of disruption of the native genome in these regions. The BCGΔ1, BCGΔ2, and BCGΔ3 deletion regions therefore provide suitable target sites for the incorporation of expression cassettes and the subsequent expression of exogenous gene products. The expression cassettes typically comprise a nucleic acid sequence under the control of a promoter. The promoter may be either constitutive or inducible. The cassette may additionally comprise a selectable marker such as an antibiotic resistance gene, a gene encoding a fluorescent marker (*e.g.* green fluorescent protein), or a gene encoding an enzymatic marker (*e.g.* ß-galactosidase).

Alternatively, genes under the control of endogenous promoters may be used as well. In one embodiment, reporter genes under the control of endogenous promoters found within the deletion sequences may be inserted at the deletion sites. These reporter genes may be utilized as an assay for antimycobacterial compounds that act by inhibiting transcription or translation of deletion sequences. Assaying for the

reporter gene product in the presence of an antimycobacterial compound provides a measure of efficacy of that compound in upregulating or downregulating deletion sequence genes. Methods of use of mycobacterial reporter gene assays to screen for drug activity are described by Cooksey *et al.*, *Antimicrob. Agents Chemother.*, 37: 1348-1352 (1993), and Jacobs *et al.*, *Science*, 260: 819-822 (1993) which are incorporated herein by reference.

EXAMPLES

The following examples are offered by way of illustration, not by way of limitation.

Example 1

Identification of Virulence-Attenuating Deletions

Bacterial Culture

All strains of Mycobacteria used in this study were maintained in 7H9 (Difco, Detroit Michigan, USA) media supplemented with OADC (BBL) or were grown on 7H11 agar supplemented with oleic acid albumin dextrose complex (OADC). *Escherichia coli* (strain DH5α or NM554) was used as a host for all recombinant plasmids and cosmids. *E. coli* was maintained in LB medium with or without agar. Carbenicillin (100 μg/ml) was used in place of ampicillin for the selection of all *E. coli* plasmids.

Extraction of High Molecular Weight DNA

High molecular weight chromosomal DNA was prepared by diluting a late log phase culture of the respective mycobacterium 1:10 into a liter of 7H9 medium containing 1.5% glycine and continuing growth for 4 to 5 days. The cells were then harvested by centrifugation, washed once in TE (pH 8.0) and resuspended in 4 ml of 25% sucrose in 10X TE. 100 μg of lysozyme was added and the preparation was incubated at 37°C for 2 hr followed by the addition of 100 μg of proteinase K and sarkosyl to a concentration of 1% weight/volume. Following overnight incubation at 65°C the mixture was extracted 4 times with chloroform isoamyl alcohol 24:1, once with phenol/chloroform (1:1), and twice again with chloroform isoamyl alcohol. The resulting high molecular weight DNA was then run on a CsCl gradient as described by

Hull *et al. Infect. Immun.*, 33: 933-938 (1981), which is incorporated herein by reference, and subsequently dialyzed against 4 changes of TE. BCG DNA was physically sheared by passage through a 22 gauge needle until an average size of 3-10 kb was obtained (20-25 passages). This DNA was then biotinylated using photobiotin (Clonetech, Palo Alto, California, USA) according to the method of Straus and Ausubel, *Proc. Natl. Acad. Sci. USA*, 87: 1889-1893 (1990), which is incorporated herein by reference.

DNA Subtraction

DNA subtraction was carried out between virulent *M. tuberculosis* H37Rv and avirulent BCG. H37R chromosomal DNA was selected because it was the most readily available chromosomal DNA from a virulent strain. In addition, *M. bovis* and *M tuberculosis* H37Rv are highly homologous.

M. bovis/M. tuberculosis specific probes were generated by the method of Straus and Ausubel, *supra.* with the following modifications. Sheared and biotinylated BCG DNA was used in a 10:1 excess for each round of subtraction. Wild type *M. tuberculosis* H37Rv DNA was digested with Sau3A to an average size of 1 kb. Hybridization conditions were 1M NaCl and 65 °C for 18 hours. Following five cycles (successive denaturation and reassociations) of subtraction, Sau3A1 adaptors (GACACTCTCGAGACATCACCGTCC and GATCGGACGGTGATGTCTCGAGAGTG were ligated to the subtraction product and amplified in a PCR reaction for 35 cycles (30 sec at 95°C, 30 sec at 55°C, and 3 min at 72°C). The *M. tuberculosis/M. bovis* specific probes were radiolabeled by using one strand of the adaptor (GACACTCTCGAGACATCACCGTCC) as a primer and labeling with ^{32}P dCTP using the Klenow fragment of DNA polymerase.

An *M. bovis* cosmid library was constructed in the BamH1 site of sCOS (Stratagene, La Jolla California, USA) with subsequent *in vitro* packaging and infection of *E. coli* strain NM554 (Stratagene). 600 colonies were picked to Nytran circular membranes and the membranes prepared according to the method of Grunstein and Hogness, *Proc. Natl. Acad. Sci. USA*, 72: 3961 (1975), which is incorporated herein by reference. These filters were then probed using the BCG subtracted probe and positive clones selected for further analysis. Cosmid DNA was prepared from selected clones by the method of Birnboim and Doly, *Nucleic Acids. Res.*, 7: 1513 (1973) which is

incorporated herein by reference. Restriction fragments that hybridize with the MTB/MBV specific probe were further subcloned into pGEM7z or pGEM5z (Promega, Madison, Wisconsin, USA) for deletion analysis.

Plasmid DNA for DNA sequencing was prepared using Qiagen minicolumns (Qiagen Inc. Chatsworth California, USA) and sequenced by the method of Henikoff, *Gene*, 28: 351-359 (1984), which is incorporated herein by reference, using the Erase A Base System (Promega). DNA sequencing reactions were run using a Perkin Elmer 9600 thermocycler and analyzed on an automated ABI sequencer. Analysis and assembly of contiguous DNA sequence was done using the ABI analysis software and SeQuencher sequence analysis software by Gene Clones Corp (Ann Arbor, Michigan, USA).

Deletion Region 1 (BCGΔ1)

Sequence analysis of over 16 kb of MBV region 1 and homologous regions in BCG revealed the precise junctions for the deletion in BCG. Eight open reading frames were identified with codon usage biases matching that of known MTB and MBV genes (see map Figure 4). The potential start and stop codons and predicted maximum protein coding capacity are listed in Figure 4. Consensus ribosomal binding site sequences were found near potential start codons for seven of eight open reading frames. TBLASTN and FASTA sequence homology analysis with each potential ORF-encoded protein revealed significant homologies for 3 of 8 open reading frames in region 1.

Most notable is the ORF1C homology to an unpublished and uncharacterized sequence listed in Genbank as *M. tuberculosis* antigen esat6. A 65 base pair repeated overlapping (repeated ~2 1/2 times) sequence was also recognized within the ORF1C (esat6) open reading frame. Also noteworthy are the significant homologies identified between ORF1H and bacterial serine proteases including *B. subtilus* subtilisin. Of the eight recognized open reading frames, four (ORFs 1B, 1C, 1D, and 1E) are located entirely within the 9 kb region deleted in BCG. One ORF traverses the BCG deletion junction in virulent *M. bovis*.

DNA probes from the 9 kb deletion in region 1 demonstrated that this region is absent in all BCG substrains and present in all virulent MBV and MTB strains tested. Furthermore, restriction fragment patterns observed in Southern blot analysis

with region 1 probes are non-polymorphic and identical in virulent MBV and MTB. This region has far fewer direct and indirect repeats than the regions 2 (BCGΔ2) and 3 (BCGΔ3) characterized below.

The sequence of a small region, estimated to be less than 20 bp between basepair coordinates 10654 and 10664 in region 1 has been recalcitrant to automated sequencing. Therefore, pending sequence confirmation, the base pair coordinates given in the region 1 map (Figure 4) are approximations. The precise sequence determination is likely to effect the Orf1E open reading frame.

Deletion Region 2 (BCGΔ2)

Sequence analysis of over 15 kb of MBV region 2 and homologous regions in BCG revealed the precise junctions for an 11 kb deletion in BCG. Thirteen open reading frames were identified with codon usage biases matching that of known MTB and MBV genes (see map Figure 5). The potential start and stop codons and predicted maximum protein coding capacity are also shown in Figure 5. Candidate consensus sequences resembling ribosomal binding sites were found near potential start codons for eight open reading frames. Of the thirteen open reading frames recognized in BCGΔ2, nine are located entirely within the 11 kb region deleted in most BCG strains while ORF2B2 and ORF2I traverse the deletion junctions.

TBLASTN and FASTA sequence homology analysis with each potential ORF-encoded protein revealed significant homologies for five open reading frames in BCGΔ2. A protein encoded by ORF2C exhibits striking similarity to the *E. coli* iciA protein which is thought to play a role in inhibiting and regulating the initiation of chromosomal replication. The iciA protein product is a member of the large LysR family of transcriptional regulatory proteins. Orf2F is highly homologous to an *S. typhimurium* ribonucleotide diphosphate reductase and a region of the *E. coli* and *S. typhimurium* proUVWX operon. Orf2H was found to have significant homology to *E. coli* and *S. typhimurium* permeases involved in aromatic amino acid transport and a eukaryotic cell retroviral receptor.

The Orf2G encoded protein was identical to the MTB mpt64 gene previously thought to encode a secreted antigen which is specifically expressed by MTB

and not BCG strains. Recent analysis of mpt64 expression revealed that three BCG substrains do express mpt64 (Moreau, Tokyo, Russian). Probes specific for mpt64 or other non-repetitive parts of region 2 hybridized to all MTB strains tested and the same three BCG substrains shown to express mpt64. Of interest is the finding that these three BCG substrains are derived from the original Pasteur strain prior to 1925. The current Pasteur strain and all strains derived from the original Pasteur strain after 1925, including the Connaught strain used in the subtractive analysis in this study, are deleted in the 11 kb DNA segment contained within BCGΔ2. These data indicate that an additional mutational event deleting the 11 kb segment of region 2, occurred in the BCG Pasteur strain sometime after 1925.

Southern blot analysis with probes from different segments of region 2 revealed a repetitive element located within a 2 kb segment (8-10 kb) of region 2. This repetitive element is ubiquitous in all tubercle bacilli tested. This element provides a marker suitable for RFLP analysis of mycobacterial strains.

Deletion Region 3 (BCGΔ3)

Sequence analysis of the almost 11 kb region 3 sequence and comparison to a homologous region in BCG precisely identified the deletion junctions for BCG. Twelve potential open reading frames were recognized in the region 3 sequence, seven of which are entirely located within the 9 kb region deleted in BCG. At least 9 ORFs in BCGΔ3 exhibit codon usage preferences comparable to that of the tubercle bacilli. Sequence homology analysis of presumptive protein sequences encoded by six open reading frames in region 3 revealed highly significant homology to listed sequences. Orfs3B, 3D, and 3E exhibit homology to phage sequences, suggesting a phage derivation for 4 or more kb of DNA in region 3. Homology to putative open reading frames in two *M. leprae* cosmids was also observed including homology to a putative *bid* gene encoding a protein involved in biotin synthesis. Also of interest was homology between ORF3A and an MTB sequence (*mce*) associated with cell invasion and intracellular survival.

Southern blot analysis with segments of region 3 deleted in BCG revealed that prototype lab strains of virulent MBV and MTB all carry deletion region 3 DNA. However, clinical isolates from PHRI are highly polymorphic or deleted in region 3.

This region contains many large direct and indirect repeats and, as mentioned above, at least 2 ORFs are homologous to phage sequences including homology to DNA invertases or recombinases. The repetitive nature of this region and the possible presence of a DNA recombinase could explain the polymorphisms observed in this region.

The sequence of a small region, estimated to be much less than 200 bp and located close to 9400 bp in Figure 3, was recalcitrant to automated sequencing and remains to be determined. Therefore, the base pair coordinates given in the region 3 map (Figure 6) 3' to the 9kb marker are approximations. The precise sequence determination of region is likely to effect the length of open reading frames 3H and 3L.

The foregoing subtractive analysis identified 3 regions in virulent *M. bovis* and *M. tuberculosis* prototype strains which are deleted in the avirulent BCG strain. The deletion located in region 2 may not have arisen in the original BCG Pasteur strain as this region is only deleted in strains derived from the original Pasteur strain after 1925. Region 3 is present in virulent MTB and MBV lab prototype strains (H37Rv, Erdman) and is highly polymorphic and at least partially deleted in the majority of MTB clinical isolates tested. Region 1 is apparently conserved and intact in all virulent MBV and MTB strains tested to date while all avirulent BCG strains tested to date are missing approximately 9kb from region 1.

Example 2

Screening and Identification of an Avirulent Mycobacterium

The ^{32} P labeled subtraction probe obtained in Example 1, was used to probe EcoRI and BamHI restricted chromosomal DNAs from BCG Connaught, *Mycobacterium bovis*, and various strains of *Mycobacterium tuberculosis* in a Southern blot. The hybridization was performed at 70°C in 6X SSC overnight.

The resulting Southern blot is illustrated in Figure 8. The probe showed no labeling of BCG reflecting the presence of all three deletions, while the other strains were labeled.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. A marker for an avirulent mycobacterium, said marker comprising a first nucleic acid that specifically hybridizes under stringent conditions with a second nucleic acid or a complement of said second nucleic acid where said second nucleic acid or complement of said second nucleic acid is selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3.

2. The marker of claim 1, wherein said marker specifically hybridizes under stringent conditions to a nucleic acid from BCG, but not to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or where said marker specifically hybridizes under stringent conditions to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, but not to a nucleic acid from BCG.

3. The marker of claim 2, wherein said marker comprises a subsequence of a nucleic acid where said nucleic acid is selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3.

4. The marker of claim 2, wherein said marker is selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3.

5. The marker of claim 2, wherein said marker comprises a nucleic acid having at least 90 percent sequence identity with a sequence selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3.

6. The marker of claim 2, wherein said marker comprises a radioactive nucleotide probe.

7. The marker of claim 2, wherein said subsequence is a sequence selected from an open reading frame of a deletion, said deletion being selected from the group consisting of BCGΔ1, BCGΔ2, BCGΔ3.

8. A polypeptide encoded by a subsequence of a deletion sequence selected from the group consisting of BCGΔ1, BCGΔ2, and BCGΔ3.

9. The polypeptide of claim 8, wherein the subsequence is selected from an open reading frame (ORF) of a deletion, said deletion being selected from the group consisting of BCGΔ1, BCGΔ2, BCGΔ3.

10. An antibody that binds specifically to the polypeptide of claim 8.

11. A recombinant cell comprising a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of said second nucleic acid where said second nucleic acid or complement of said second nucleic acid is selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3.

12. The recombinant cell of claim 11, wherein the cell is a Mycobacterium.

13. The cell of claim 11, wherein the cell expresses a polypeptide encoded by an intact open reading frame from BCGΔ1, BCGΔ2, and BCGΔ3.

14. The cell of claim 11, wherein said cell is a mycobacterium having one or more deletions in the genomic regions selected from the group consisting of BCGΔ1, BCGΔ2, and BCGΔ3, wherein said deletions result in the attenuation of an otherwise virulent strain of mycobacterium and wherein said deletions are present in up to two of said regions.

15. The mycobacterium of claim 14, wherein said deletions comprise a deletion selected from the group consisting of BCGΔ1, BCGΔ2, and BCGΔ3.

16. A method of distinguishing between an attenuated and a virulent mycobacterium, said method comprising detecting the presence or absence of a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of said second nucleic acid where said second nucleic acid or complement of said second nucleic acid is selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3.

17. The method of claim 16, wherein said first nucleic acid specifically hybridizes under stringent conditions to a nucleic acid from BCG, but not to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or where said first nucleic acid specifically hybridizes under stringent conditions to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, but not to a nucleic acid from BCG.

18. The method of claim 17, wherein said first sequence is amplified prior to detection.

19. The method of claim 17, wherein said first sequence is amplified by the polymerase chain reaction.

20. A method of claim 17, wherein said detecting comprises a Southern blot.

21. A method of claim 17, wherein said detecting comprises detecting a polypeptide encoded by said first nucleic acid.

22. The method of claim 21, wherein the polypeptide is encoded by an intact open reading frame of a nucleotide sequence selected from the group consisting of BCGΔ1, BCGΔ2, and BCGΔ3.

23. The method of claim 21, wherein the polypeptide is visualized by antibody hybridization.

24. A method for determining whether an attenuated or a virulent Mycobacterium is present in a sample comprising:

providing a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of said second nucleic acid where said second nucleic acid or complement of said second nucleic acid is selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3; and

hybridizing said first nucleic acid to the biological sample.

25. The method of claim 24, wherein said first nucleic acid specifically hybridizes under stringent conditions to a nucleic acid from BCG, but not to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or where said first nucleic acid specifically hybridizes under stringent conditions to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, but not to a nucleic acid from BCG.

26. A method of producing an attenuated Mycobacterium species, said method comprising deleting from the genomic DNA of a virulent mycobacterium a first nucleic acid that specifically hybridizes under stringent conditions with a second nucleic acid or a complement of said second nucleic acid where said second nucleic acid or complement of said second nucleic acid is selected from the group consisting of BCGΔ1, BCGΔ2, and BCGΔ3.

Figure 1

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      |  10      |  20      |  30      |  40      |  50      |  60      |  70      |  80      |  90      | 100
   1 GAATTCCTGC GCACCCTGAT CCTGTCGCTG GTGGCAATGA CTCATCCAGA TCAGGTGAAT CTCCTGCTCA CCGACTTCAA AGGTGGTTCA ACCTTCCTGG 100
 101 GAATGGAAAA GCTTCCGCAC ACTGCCGCTG TCGTCACCAA CATGGCCGAG GAAGCCGAGC TCGTCAGCCG GATGGGCGAG GTGTTGACCG GAGAACTCGA 200
 201 TCGGCGCCAG TCGATCCTCC GACAGGCCGG GATGAAAGTC GGCGCGGCCG GAGCCCTGTC CGGCGTGGCC GAATACGAGA AGTACCGCGA ACGCGGTGCC 300
 301 GACCTACCCC CGCTGCCAAC GCTTTTCGTC GTCGTCGACG AGTTCGCCGA GCTGTTGCAG AGTCACCCGG ACTTCATCGG GCTGTTCGAC CGGATCTGCC 400
 401 GCGTCGGGCG GTCGCTGAGG GTCCATCTGC TGCTGGCTAC CCAGTCGCTG CAGACCGGCG GTGTTCGCAT CGACAAACTG GAGCCAAACC TGACATATCG 500
 501 AATCGCATTG CGCACCACCA GCTCTCATGA ATCCAAGGCG GTAATCGGCA CACCGGAGGC GCAGTACATC ACCAACAAGG AGAGCGGTGT CGGGTTTCTC 600
 601 CGGGTCGGCA TGGAAGACCC GGTCAAGTTC AGCACCTTCT ACATCAGTGG GCCATACATG CCGCCGGCGG CAGGCGTCGA AACCAATGGT GAAGCCGGAG 700
 701 GGCCCGGTCA ACAGACCACT AGACAAGCCG CGCGCATTCA CAGGTTCACC GCGGCACCGG TTCTCGAGGA GGCGCCGACA CCGTGACCCG CGCCGGCGAC 800
 801 GATGCAAAGC GCAGCGATGA GGAGGAGCGG CGCCAACGGC CCGCGCCGGC GACGATGCAA AGCGCAGCGA TGAGGAGGAG CGGCGCGCAT GACTGCTGAA 900
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15301 GGCATCCCGG TCTGGCCGAT ACCGCCGACG CTTAACACCA GCGGTGCGTA  CCACGCCGGG GTGACAACGG TCTGCACATT GTTCCAGCCG CGTGGGTCGC 15400
15401 CGGGTGTGGA CGGGTCCGGC GCCGGATTCT GTACGCAATC GCCACCGGTG  TTGCCGGCCG CGACCACCAC CACCACGCCT TTGACGTTGA CCGCATAGTC 15500
15501 GATGGATGCA CCCAGTGAGG TTTCATCGAT CGGCCTGCTC ACCTTGTAGC  AGGCGGCTTC ACTGATGTTG ATCACACCCA CGCCGAGGTT GGCGGCGTGC 15600
15601 ACCACGGCGC GGGCAAGACT GCGGATGGAA CCGGCGGCCG GGGTGGCGTT  GGGGTCATTC GGGTTGGCTT GTGAGCCGAC CGGTTCGAAG GCCTCAGACG 15700
15701 TCTGACGTAG CGAGAGCAGT CGAGCGTCGG GCGCGACGCC GACGAACCCG  TCGGTGGGCG CGGGCCGGCC CGCGATGATG GATGCTGTGA GAGTCCCATG 15800
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16701 ATCTCGACGA TCTCCACCGG CGAATAAGAC TCGAACCGCA GCTTTCGGTT  GAACCGGCCA GCCAAACCCG GGTTCACGGT GAGGAATTCG GTACCCCGGG 16800
16801 TTCGAAATCG ATAACTTGGA TCCGGAGAGC TCCCAACGCG TTGGATGCAT  AGCTTGAGTA TTCTATAGTG TCACCTAAAT ACTTG                 16885
          |  10      |  20      |  30      |  40      |  50      |  60      |  70      |  80      |  90      | 100
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Figure 2


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      |  10      |  20      |  30      |  40      |  50      |  60      |  70      |  80      |  90      | 100
    1 GGATCCTCGG ACTGGCCGCG GTCGTGCTTG TGCACGAGTT CACCGAGGTC ATCGTCATCG CCAACGGCGT GCGGGCCGGA CGCATCAAAC CACTTGCCGG 100
  101 GCCACCCAAG ACACCTGATC GGACTATCCC GGGGTAGCGA CGCGCGGAAT CGTGGAGTGT GTTTGGACCA GCAATAGCGT CACTGTGACG AAACAGCCGC 200
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  301 TCGGCATGAG TCGGTCCGAG TTCTTCACGA AGGCTGCGCA GCGCTACCTG CACGAGCTGG ACGCCCAATT GCTCACGGGC CAGATCGACA GGGCTCTAGA 400
  401 GAGCATCCAT GGCACCGACG AAGCGGAGGC CCTCGCCGTG GCCAACGCAT ACCGCGTGCT AGAAACCATG GACGATGAGT GGTGATTAGT CGTGCCGAGA 500
  501 TCTACTGGGC TGACCTCGGG CCGCCATCAG GCAGTCAGCC GGCGAAGCGC CGCCCGGTGC TCGTAATCCA GTCAGATCCG TACAACGCAA GTCGCCTTGC 600
  601 CACTGTGATC GCAGCGGTGA TCACGTCCAA TACGGCGCTG GCGGCAATGC CCGGCAACGT GTTCTTGCCC GCGACCACAA CGCGACTGCC ACGTGACTCG 700
  701 GTCGTCAACG TCACGGCGAT TGTCACGCTC AACAAGACTG ACCTCACCGA CCGAGTTGGG GAGGTGCCAG CGAGCTTGAT GCACGAGGTT GACCGAGGAC 800
  801 TTCGTCGCGT ACTGGACCTT TGACACTGCG CCACGCGACA ATTCGTCACG GTGACGTTCC TGCTTGGTGT AAGCCCCCCC GCCGGGGGAA CTACTCGCCG 900
  901 GAGGTGGTGT TGTGGGCAGG CTTGAGGGCA AGGTTGCATT CATTACGGGC GTGGCTCGGG GTCAAGGCCG TTCGCATGCG GTCCGCCTAG CCGACGGCCA 1000
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Figure 2 continued, page 4 of 4

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15101 CGCGACCGGC GAGGTTGCCA CCATGGTCGT CGAAGCAACT TGCTGCTAAC  GAGCCTGTAG TTTTGCCAGC CCCCACTCGC GCTTTGTCTG CAGGTTTTCA 15200
15201 GGCTCAGCGA CGGCTCATGT CGTTGCGCAC GGCGAATTC                                                              15239
          |  10     |  20     |  30     |  40     |  50     |  60     |  70     |  80     |  90     |  100
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Figure 3

WO 96/25519

PCT/US96/01938

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      |  10      |  20      |  30      |  40      |  50      |  60      |  70      |  80      |  90      | 100
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  701 GCATGTCACG GCTAAGTGGC TACCTGACCC CCCAAGCGCG GGCCACCTTT GAAGCCGTGC TAGCCAAACT GGCCGCCCCC GGCGCGACCA ACCCCGACGA 800
  801 CCACACCCCG GTCATCGACA CCACCCCCGA TGCGGCCGCC ATCGACCGCG ACACCCGCAG CCAAGCCCAA CGCAACCACG ACGGGCTGCT GGCCGGGCTG 900
  901 CGCGCGCTGA TCGCCTCCGG GGAACTGGGC CAACACAACG GTCTTCCCGT CTCGATCGTG GTCACCACCA CCCTGACCGA CCTGCAAACC GGCGCCGGCA 1000
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11501 CCCGCACCGT AGCAATGCGC GCCAGCGCGG ACCGATTCGA GGCTGCCACC AACCCGGGCG GGTCCGGCCA GCTGCCGATC ACCAGCCCTG CACATGAAAC 11600
11601 CTGTTGTGCA GCAAGCGCTT CCAACGTCAA CTTGGTGTGG TTGAGGGTGC CCAGGTCCGC GGTGACCACC ACCAAAGCCG CGGCGGCCAC GTCGACGGCG 11700
11701 ACATCGCGCA GCGTGACGCC CGGCTCGGCG AGTTCGACCA GCAGCCCGCC CGCCCCCTCG ACGAGGGTCA ACCGCCCGGG ACGGTCCAGG TCTGCGATCA 11800
11801 GCCGCACGAT CTGATCGCGG GCGGGCAACG CCATCCCGGC GTGTTCGGCG GCGGCGGCCG GGGCCATCGG CTGCGGATAT CGCGCCAAGC CGGCCAGCTG 11900
11901 GGTCACCCCG GCCAACCGGC CGACCTCGGC GAGGTCGTCG TCACCGCGGG CGGTGCCGGT CTGAACGGGC TTGCACACCG CCACGTCGAT GCCGGCCTGA 12000
12001 CGTGCGGCCG ACGCCAGCGC CGCGCAGACG ACCGTCTTGC CGACCCCCGT GCCGGTCCCG GTGACGACCA GGATCGTCAA CGGCGCGCCA CGGCGAGAAC 12100
12101 ATCCGTCAGC ACCCGCCGGG CCAGCTCGAG CTCGCCGGCG TTCAGCGATG CGCGCGCGGT CAGCCGCAGC CGCGACGTAC CCGCGGGCAC CGTCGGCGGC 12200
12201 CGGAAGCAGC CCACCTTGAC CCCGGCGTCC AGGCAGGCCG CCGCGGCGGC CACTGCCGAC TCCGGCTCGC CCAGGATCAC CGACACCATC GCCGAGTCCG 12300
12301 GCACCGCAGC CACACCGCAC ATCCGCGCAA GTTCACCAGC GTGGTTGAGC ACCGCCTGCG ATCGCCACGG CTCGGCCTGC AAGACGCGCA GCGCGGCCCG 12400
12401 TGCGGCACCT TC                                                                                          12412
        | 10     | 20     | 30     | 40     | 50     | 60     | 70     | 80     | 90     | 100
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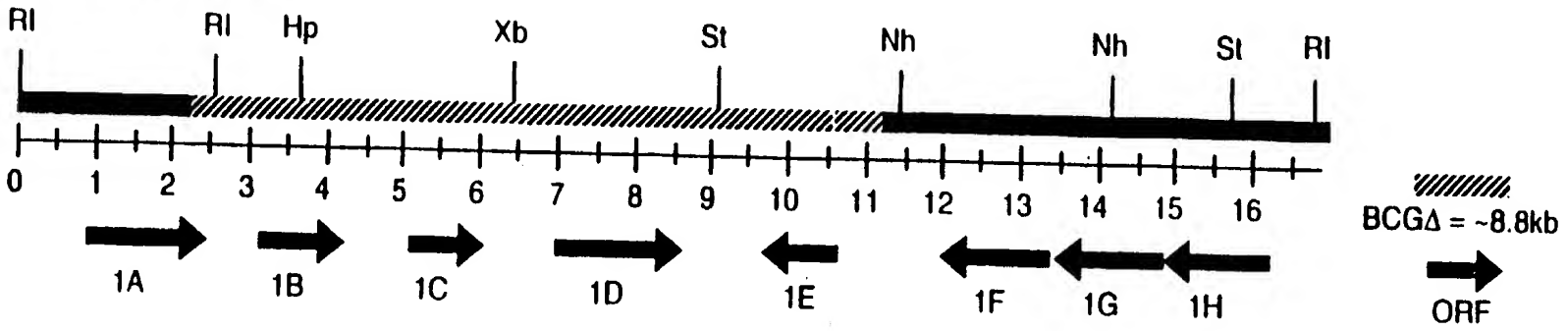
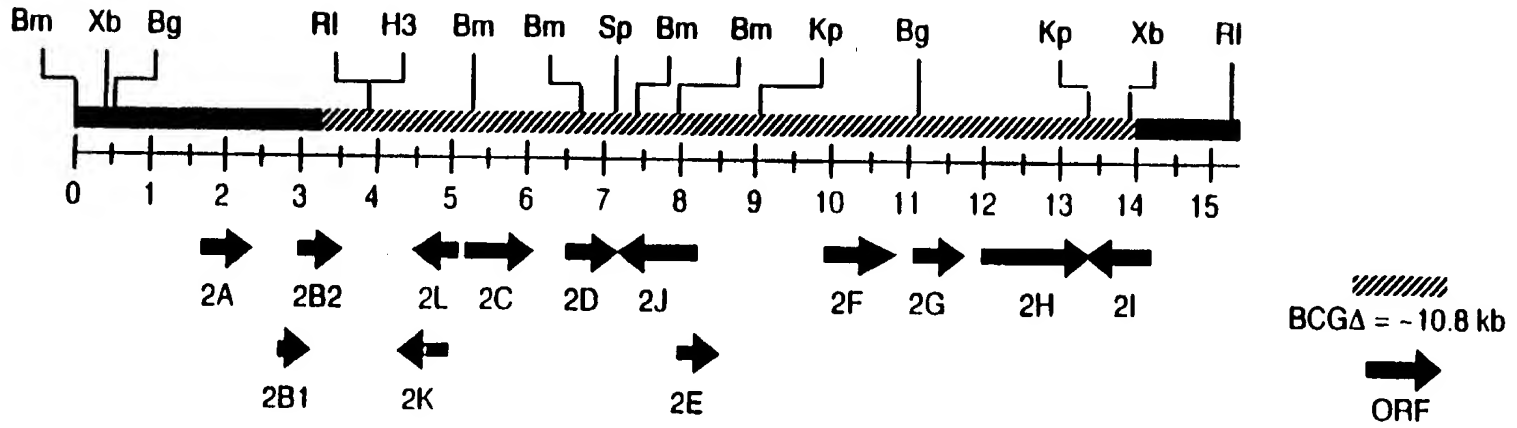
Region 1 (16.9 kb)



ORF	*M. tuberculosis* Codon Usage	ORF Size (base pairs)	Start - Stop (base pairs)	Possible Ribosome Binding Sites	Encoded Protein (max. ~kDa)	Homologies to Predicted Encoded Protein	P value	Homologue Accession #
1A	yes	1542	889 - 2433	AGGA (10)	57			
1B	yes	1071	3130 - 4203 3139 - 4203	GGA (4) GGA (9)	36	*M.leprae* aceA BCG uraA	1.4e-14 3.0e-13	Z46257 U01072
1C	yes	969	5075 - 6046	GAGG (5)	34	*M.tuberculosis* esat6	2.3e-43	X79562
1D	yes	1657	6954 - 8612	none	59			
1E	yes	954	10619 - 9663	GGA (5)	34			
1F	yes	1380	13328 - 11946	AGGA (9)	48			
1G	yes	1386	14823 - 13438 14643 - 13438 14541 - 13438	none GGA (11) AGGAGA (10)	50			
1H	yes	1368	16190 - 14820	GAA (5)	46	*B.subtilis* subtilisin Serine proteases	3.6e-16 4.8e-14	L29506 A08331

Figure 4

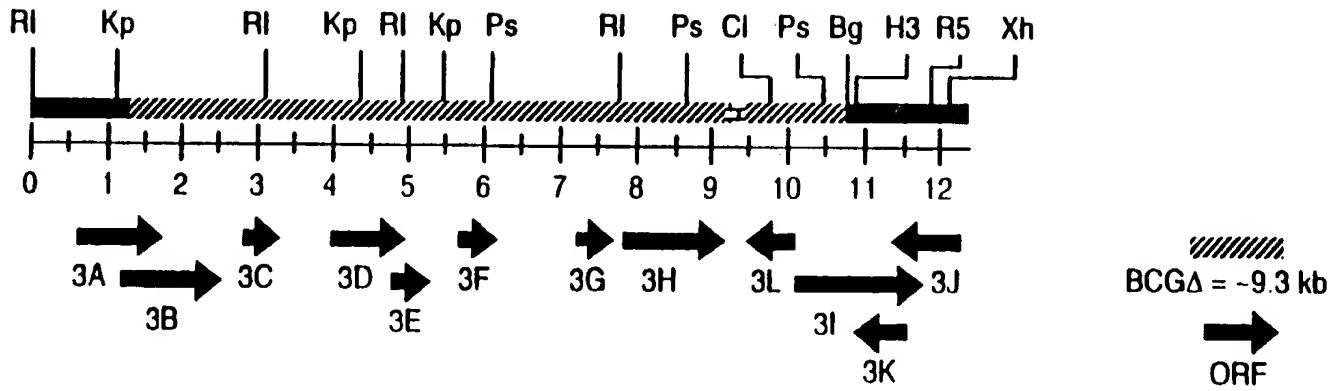
Region 2 (15.3 kb)



ORF	*M. tuberculosis* Codon Usage	ORF Size (base pairs)	Start - Stop (base pairs)	Possible Ribosomal Binding Sites	Encoded Protein (max. -kDa)	Homologies to Predicted Encoded Protein	P value	Homologue Accession #
2A	yes	558	1829 - 2386	AGGGAG (7)	25			
2B1	yes	437	2862 - 3298	AGAA (4)	16			
2B2	yes	588	3003 - 3590	none	34			
2C	yes	948	5187 - 6134 5376 - 6134	AG? (8) GGA (8)	34	*E.coli* iciA lysR family	9.9e-47 <1e-5	P24194
2D	yes	657	6561 - 7217	none	22	*Mleprae* cosmid B1620 ORF Cutinases	1.5e-7 ~4.e-5	U00015 A00975 U03393
2E	yes	522	8036 - 8560	none	19			
2F		966	9941 - 10909	AGGA (11)	37	*S.typhimurium* RNDPR proUVWX	9.9e-146 2.7e-36	X73226 X17445
2G	yes	666	11118 - 11783	AAGA (6)	24	*M.tuberculosis* mpt64	6.7e-141	A30545
2H	yes	1443	11965-13407	AG (10)	51	*E. coli* gabP permease *S. typhimurium* asp permease *T. harzianum* indal gene retroviral receptor	3.1e-11 1.4e-08 4.4e-11 2.5e-09	X65104 U04851 Z22594 X59155
2I	yes	846	14221-13376	GGAAGA (6)	31			
2J	yes	1050	8259 - 7211 7939 - 7211 7931 - 7211	GAG (10) GGAA (8) GGA (9)	35			
2K	yes	666	4992 - 4327	none	25			
2L	yes	597	5117 - 4521	AG (10)	21			

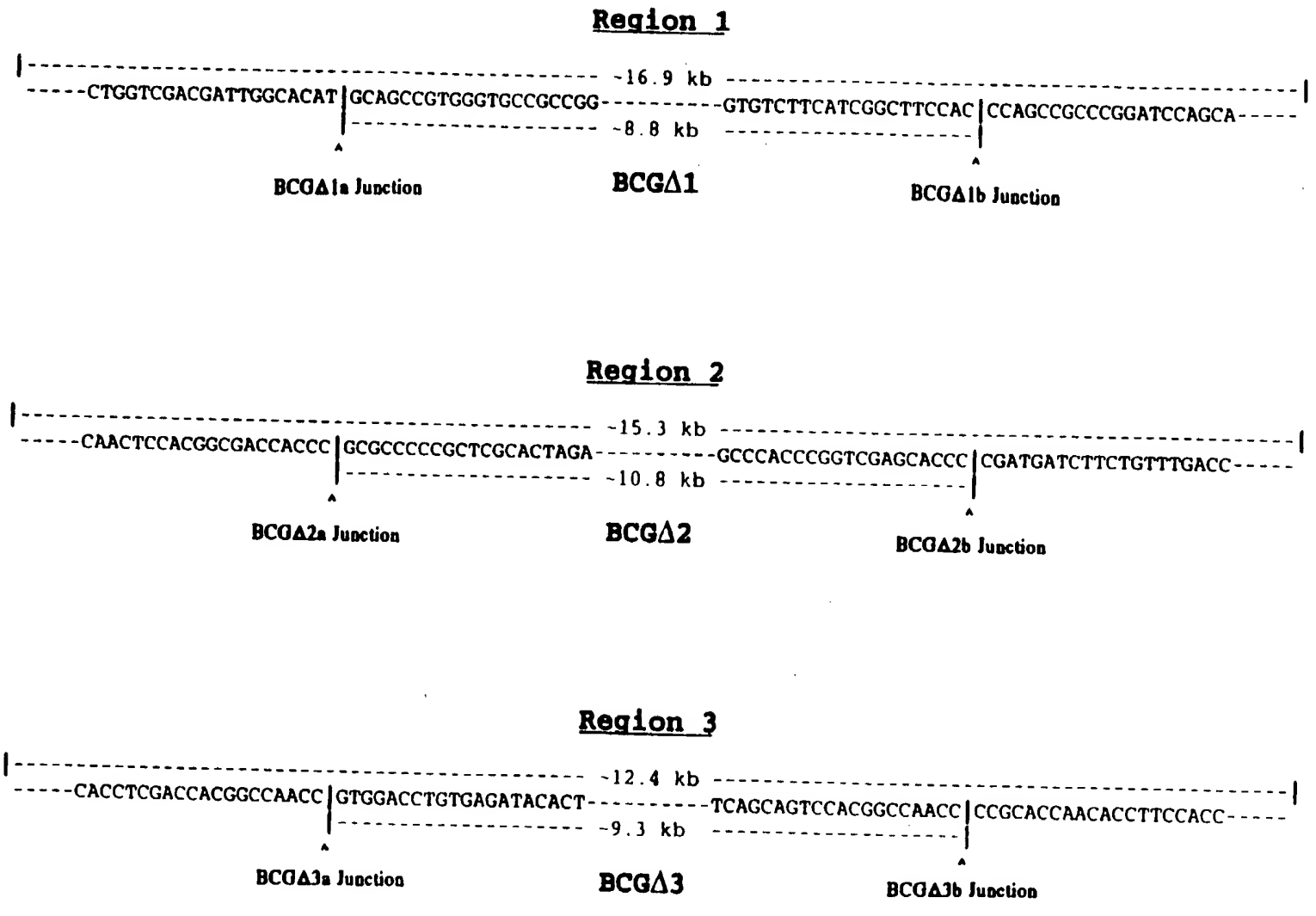
Figure 5

Region 3 (12.4 kb)



ORF	*M. tuberculosis* Codon Usage	ORF Size (base pairs)	Start - Stop (base pairs)	Possible Ribosomal Binding Sites	Encoded Protein (max. ~kDa)	Homologies to Predicted Encoded Protein	P value	Homologue Accession #
3A	yes	1143	613 - 1755	none	42	MTB mce sau3A *M. leprae* cosmid L247	2.9e-64 5.1e-13	X70901 U00021
3B	yes	1347	1214 - 2560		49	Actinophage R4 attP gene *B. subtil.* site spec. recomb. recombinases / invertases	3.0e-05 7.8e-4 8.2e-4	D90361 M29040 K00676 X01805, X07724
3C	yes	513	2820 - 3332	GGA (6)	19			
3D	no no	924	4007 - 4930 4070 - 4930	none GGA (7)	34	*S. coelicolor* phage phi-C31 early region	4.2e-26	X76288
3E	no yes	543	4795 - 5337 4915 - 5337	none GGAA (5)	21	*S. coelicolor* phage phi-C31 pglY pglZ genes	3.2e-11	X76288 L37531
3F	yes	576	5639 - 6214	GA (9)	20			
3G	yes	510	7253 - 7762 7285 - 7762	GAAGG (8)	19			
3H	yes	1330	7868 - 9197	GA (8)	47			
3I	yes	1665	10146 - 11810 10164 - 11810	GGAAG (6)	58	*M. leprae* B1170	6.2e-69	U00010
3J	yes	918	12319 - 11402	GAA (11)	30	*M leprae* bioDAYB *C. glutamicum* bioD *B.sphaericus* bioDAYB	6.9e-53 2.6e-08 1.4e-05	U00010 D14083 M29292
3K	yes	702	11594 - 10893	AGG (4)	25	*M. leprae* cosmid B1170	1.0e-81	U00010
3L	yes	660	10147 - ?? 9488	AG (10)	25			

Figure 6


Region 1
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-----CTGGTCGACGATTGGCACAT|GCAGCCGTGGGTGCCGCCGG----------GTGTCTTCATCGGCTTCCAC|CCAGCCGCCCGGATCCAGCA-----
~8.8 kb
BCGΔ1a Junction
BCGΔ1
BCGΔ1b Junction
Region 2
~15.3 kb
-----CAACTCCACGGCGACCACCC|GCGCCCCCGCTCGCACTAGA----------GCCCACCCGGTCGAGCACCC|CGATGATCTTCTGTTTGACC-----
~10.8 kb
BCGΔ2a Junction
BCGΔ2
BCGΔ2b Junction
Region 3
~12.4 kb
-----CACCTCGACCACGGCCAACC|GTGGACCTGTGAGATACACT----------TCAGCAGTCCACGGCCAACC|CCGCACCAACACCTTCCACC-----
~9.3 kb
BCGΔ3a Junction
BCGΔ3
BCGΔ3b Junction

Figure 7

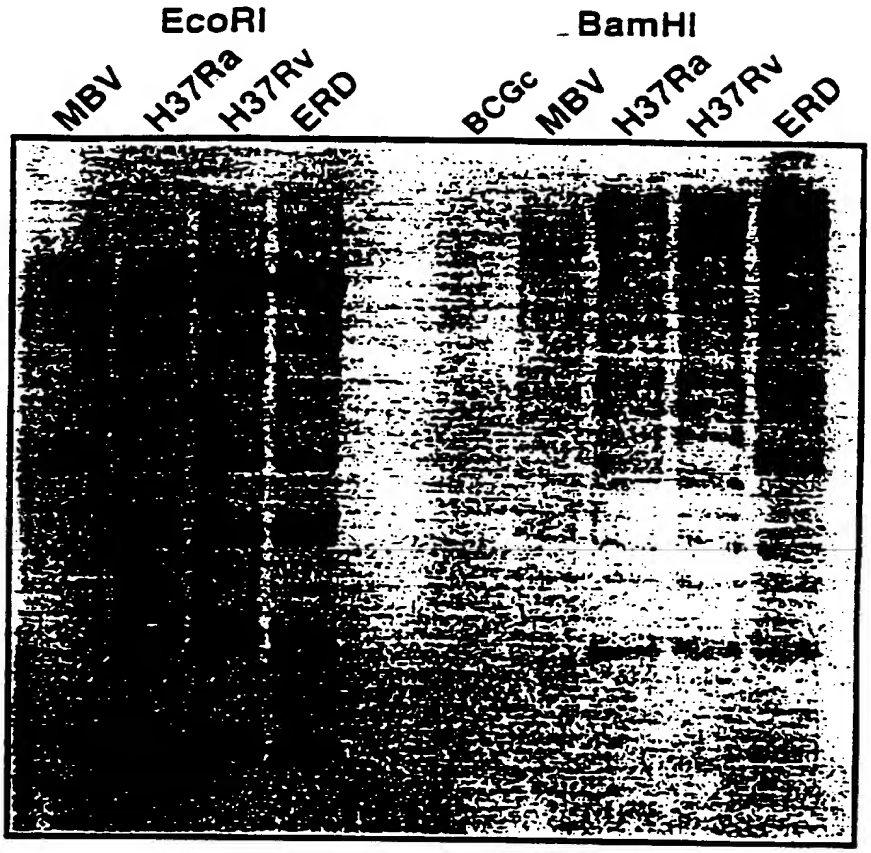

Figure 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01938

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 91.1, 91.2, 240.2, 252.3; 530/300, 350, 387.1, 388.1; 536/22.1, 23.1, 24.3, 24.32, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	Infection and Immunity, Volume 61, No. 5, issued May 1993, H. Li et al, "Evidence for absence of the MPB64 gene in some substrains of Mycobacterium bovis BCG", pages 1730-1734, see entire document.	1-10, 16, 17, 24, 25 ----- 18-23
X	JP, 1-247094 (AJINOMOTO ET AL) 02 October 1989, see entire document.	1-7
X	Infection and Immunity, Volume 59, No. 10, issued October 1991, C. Parra et al, "Isolation, characterization and molecular cloning of a specific mycobacterium tuberculosis antigen gene: identification of a species-specific sequence", pages 3411-3417, see entire document.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
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Date of the actual completion of the international search 17 APRIL 1996	Date of mailing of the international search report 29 MAY 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEFFREY FREDMAN Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01938

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Abstracts of the 1994 IDSA Annual Meeting,Clin. Infect. Dis., Volume 19, issued October 1994, R. Frothingham et al, "Sequence based strain differentiation in the Mycobacterium tuberculosis complex, including rapid identification of M. bovis BCG", page 565, see abstract 10.	1-25
X	R. GHERNA et al, "AMERICAN TYPE CULTURE COLLECTION: CATALOGUE OF BACTERIA AND PHAGES", Eighteenth edition, published 1992, pages 202 and 211, see entire document.	11-15
X ---- Y	Infection and Immunity, Volume 62, No. 4, issued April 1994, L. Pascopella et al, "Use of in vivo complementation in Mycobacterium tuberculosis to identify a genomic fragment associated with virulence", pages 1313-1319, see entire document.	1-7, 16-25 ---- 26
Y	Science, Volume 261, issued 10 September 1993, S. Arruda et al, "Cloning of an M. Tuberculosis DNA fragment associated with entry and survival inside cells", pages 1454-1457, see entire document.	1-23
X ---- Y	US,A,5,171,839 (PATARROYO) 15 December 1992, columns 5-10.	1-10 ---- 16-23
Y	Nature, Volume 256, issued 07 August 1975, C. Kohler et al, "Continuous cultures of fused cells secreting antibody of predefined specificity", pages 495-497, see entire document.	10
Y	US,A, 4,683,202 (MULLIS) 28 July 1987, see entire document.	16-22, 24, 25
Y	Genomics, Volume 4, issued 1989, D. Wu et al, "The ligation amplification reaction (LAR) amplification of specific DNA sequences using sequential rounds of template directed ligation", pages 560-569, see figure 2.	16-22, 24, 25
Y	US,A, 4,410,660 (STRAUS) 18 October 1983, columns 14 and 15.	23
Y	Gene, Volume 131, issued 1993, A. Kinger et al, "Identification and cloning of genes differentially expressed in the virulent strain of mycobacterium tuberculosis", pages 113-117, see page 114, column 2.	1-26
X,P	WO,A2,95/17511 (JACOBS ET AL) 29 June 1995, see entire document.	1-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01938

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,E	J. Bacteriol., Volume 178, No. 5, issued March 1996, G. Mahairas et al, "Molecular analysis of genetic differences between mycobacterium bovis BCG and virulent M. bovis", pages 1274-1282, see entire document.	1-26
Y, P	Microbiology, Volume 141, issued 1995, J. Rodriguez et al, "Species-specific identification of mycobacterium bovis by PCR", pages 2131-2138, see entire document.	1-7, 16-22, 24, 25
X ---- Y	Hybridoma, Volume 13, No. 1, issued 1994, A. Arya et al, "Production and characterization of new murine monoclonal antibodies reactive to mycobacterium tuberculosis", pages 21-30, see page 27, table 1.	8-10 ---- 16-23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01938

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12Q 1/68; G01N 33/53; C12P 19/34; C12N 5/10, 1/21; C07K 5/00, 14/00, 16/00; C07H 21/02, 21/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.1, 91.1, 91.2, 240.2, 252.3; 530/300, 350, 387.1, 388.1; 536/22.1, 23.1, 24.3, 24.32, 24.33

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, CAPLUS, WPIDS
search terms: mycobacter?, tubercul?, bovis?, BCG, calmette, guerin, DNA, RNA, oligo, nucleic, oligonucleotide, hybridi?, probe, primer, amplif?, PCR, polymerase chain, ligase chain, LCR, attenuat?, immunoassay, antibod?, monoclon?, polyclon?, protein, peptide,
antigen, virulenc?, infect?

CORRECTED VERSION*

PCT

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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A1

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(30) Priority Data:
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(74) Agents: HUNTER, Tom et al.; Townsend and Townsend and Crew, Steuart Street Tower, One Market, San Francisco, CA 94105-1492 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: VIRULENCE-ATTENUATING GENETIC DELETIONS

(57) Abstract

The present invention provides specific genetic deletions that result in an avirulent phenotype of a mycobacterium. These deletions may be used as phenotypic markers of providing a means for distinguishing between disease-producing and non-disease producing mycobacteria.

* (Referred to in PCT Gazette No. 49/1996, Section II)

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VIRULENCE-ATTENUATING GENETIC DELETIONS

BACKGROUND OF THE INVENTION

Mycobacterium tuberculosis (MTB) infects over ten million people each year and kills over three million, making it the infectious agent causing the greatest mortality worldwide. In an effort to combat *Mycobacterium tuberculosis*, vaccination programs using a viable attenuated strain of *Mycobacterium bovis* called bacille Calmette-Guérin (BCG) have been established in more than 120 countries over the course of the last 5 decades. Although widely used and considered safe enough to administer to infants, the BCG vaccine is controversial for two principle reasons: 1) Efficacy for BCG vaccines against tuberculosis has varied from 0-85% in different clinical trials; and 2) Immunization with BCG sensitizes vaccinees to the tubercular antigens used in the tuberculin skin test, confounding attempts to discriminate between BCG immunization and TB infection. For these two reasons, especially the latter, BCG is not used in the United States where surveillance with the tuberculin test is preferred.

The original Pasteur BCG strain was developed by multiple (230 times) serial passages in liquid culture. BCG has never been shown to revert to virulence in animals indicating that the attenuating mutations in BCG are stable deletions and/or multiple mutations which cannot revert. However, the mutations which arose during serial passage of the original BCG strain have never been identified. Moreover, recent efforts to genetically complement BCG virulence with genomic libraries of virulent tubercle bacilli have also been unsuccessful again suggesting that multiple unlinked mutations are responsible for the attenuation of BCG virulence. The antigenicity of BCG and the characteristics leading to its avirulence are thus poorly understood.

SUMMARY OF THE INVENTION

The present invention provides specific genetic deletions that account for the avirulent phenotype of the bacille Calmette-Guérin (BCG) strain of *Mycobacterium bovis*. These deletions may be used as phenotypic markers of providing a means for distinguishing between disease-producing and non-disease producing mycobacteria.

In a preferred embodiment, this invention provides for nucleic acid sequences that are markers for avirulent or virulent mycobacteria. The sequences uniquely characterize the presence or absence of deletions that result in an avirulent phenotype. More specifically the sequence are either deletion junction sequence or deletion sequences or subsequences within deletion junction sequences or deletion sequences. Thus, this invention provides for a marker for an avirulent mycobacterium comprising a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of the second nucleic acid where the second nucleic acid or its complement includes BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3. In a particularly preferred embodiment, the marker specifically hybridizes under stringent conditions to a nucleic acid from BCG, but not to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or alternatively, the marker specifically hybridizes under stringent conditions to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, but not to a nucleic acid from BCG. The marker may be the full length BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3 or a subsequence within any of these regions. The marker may also include a nucleic acid having at least 80%, preferably 90%, more preferably 95%, and most preferably 98% percent sequence identity with BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, or BCGΔ3. The marker may also include a sequence selected from an open reading frame of a the deletion sequences BCGΔ1, BCGΔ2, BCGΔ3. Suitable open reading frames are indicated in Figures 4, 5, and 6.

The above described marker may be a probe. The probe may be labeled by a number of means including, but not limited to radioactive, fluorescent, enzymatic, and colorimetric labels.

In another embodiment, this invention provides for polypeptides encoded by a subsequence of the BCGΔ1, BCGΔ2, or BCGΔ3 deletions. In particular, the subsequence may be selected from an open reading frame (ORF) present in one of these deletion sequences. This invention also provides for monoclonal or polyclonal antibodies that

specifically bind polypeptides encoded by one or more subsequences of the BCGΔ1, BCGΔ2, or BCGΔ3 deletions.

In still another embodiment, this invention provides for a recombinant cell comprising a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of the second nucleic acid where the second nucleic acid or its complement is BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, or BCGΔ3. The recombinant cell may be a mycobacterium. The recombinant cell may express a polypeptide encoded by any of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3. More preferably, the recombinant cell expresses a polypeptide encoded by an intact open reading frame present in any of these regions. The cell may also be a mycobacterium having one or more deletions in the BCGΔ1, BCGΔ2, or BCGΔ3 genomic regions where the deletions result in the attenuation of an otherwise virulent strain of mycobacterium and wherein the deletions are present in up to two of the genomic regions.

In still yet another embodiment, this invention provides a method of distinguishing between an attenuated and a virulent mycobacterium. The method involves detecting the presence or absence of a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of the second nucleic acid where the second nucleic acid or its complement is BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, or BCGΔ3. The first nucleic acid may include any of the markers described above. A particularly preferred marker is one that specifically hybridizes under stringent conditions to a nucleic acid from BCG, but not to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or alternatively, that specifically hybridizes under stringent conditions to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, but not to a nucleic acid from BCG. The method may involve amplifying either the first nucleic acid by any of a number of methods including, for example, polymerase chain reaction. The detection may involve detecting the first nucleic acid, for example, as in a Southern blot, or alternatively, detecting a polypeptide encoded by the first nucleic acid. More specifically, the polypeptide may be

a encoded by an open reading frame (ORF) selected from BCGΔ1, BCGΔ2, or BCGΔ3. The polypeptide may be visualized by a number of means well known to those of skill in the art including antibody hybridization such as direct or indirect binding of labeled antibody.

This invention additionally provides a method for determining whether an attenuated or a virulent Mycobacterium is present in a sample. This method involves providing a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of the second nucleic acid where the second nucleic acid or its complement is BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, or BCGΔ3; and hybridizing the first nucleic acid to the biological sample. The first nucleic acid may include any of the markers described above. A particularly preferred marker is one that specifically hybridizes under stringent conditions to a nucleic acid from BCG, but not to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or alternatively, that specifically hybridizes under stringent conditions to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, but not to a nucleic acid from BCG. The method may involve amplifying either the first nucleic acid by any of a number of methods including, for example, polymerase chain reaction. The detection may involve detecting the first nucleic acid, for example, as in a Southern blot, or alternatively, detecting a polypeptide encoded by the first nucleic acid. More specifically, the polypeptide may be a encoded by an open reading frame (ORF) selected from BCGΔ1, BCGΔ2, or BCGΔ3. The method may also include detecting the hybridized first nucleic acid. This may involve direct detection of a label or additionally involve an amplification step and subsequent detection of the amplified product.

Finally, this invention provides a method of producing an attenuated-virulence mycobacterium. This method involves deleting from the genomic DNA of a virulent mycobacterium a first nucleic acid that specifically hybridizes under stringent conditions with a second nucleic acid or a complement of said second nucleic acid where said second nucleic acid or complement of said second nucleic acid is selected from the group consisting of BCGΔ1, BCGΔ2, and BCGΔ3. The first nucleic acid may be BCGΔ1, BCGΔ2, or BCGΔ3, or alternatively, it may be a promoter, other control element or an open reading frame from BCGΔ1, BCGΔ2, or BCGΔ3.

Definitions

Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

The phrase "specifically detect" as used herein refers to the process of determining that a particular subsequence is present in a DNA sample. A DNA sequence may be specifically detected through a number of means known to those of skill in the art. These would include, but are not limited to amplification of the particular target sequence through polymerase chain reaction or ligase chain reaction, hybridization of the sequence to a labeled probe, and binding by labelled ligands or monoclonal antibodies. For a discussion of various means of detection of specific nucleic acid sequences see Perbal, B. *A Practical Guide to Molecular Cloning*, 2nd Ed. John Wiley & Sons, N.Y. (1988) which is incorporated herein by reference.

The phrase "select subsequence" is used herein to refer to a particular DNA subsequence that is of interest. It is often a predetermined or known sequence of nucleic acid bases. A select subsequence is typically chosen because of a unique sequence identity. Typically a select subsequence is targeted for DNA amplification and often is useful as a specific marker for the presence of a particular gene or a deletion of a particular nucleic acid sequence.

The term "oligonucleotide" refers to a molecule comprised of two or more deoxyribonucleotides or ribonucleotides. Oligonucleotides may include, but are not limited to, primers, probes, nucleic acid fragments to be detected, and nucleic acid controls. Oligonucleotides include naturally occurring nucleotides, chemically modified naturally occurring nucleotides and synthetic nucleotides. The exact size of an oligonucleotide depends on many factors and the ultimate function or use of the oligonucleotide.

The term "primer" refers to an oligonucleotide, whether natural or synthetic, capable of acting as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced, *i.e.*, in the presence of four different nucleoside triphosphates and an agent for polymerization (*i.e.*, DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. A primer is preferably a single-stranded oligodeoxyribonucleotide.

The appropriate length of a primer depends on the intended use of the primer but typically ranges from 15 to 25 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template.

The phrase "PCR primers competent to amplify" as used herein refers to a pair of PCR primers whose sequences are complementary to DNA subsequences immediately flanking the DNA subsequence (target sequence) which it is desired to amplify. The primers are chosen to bind specifically those particular flanking subsequences and no other sequences present in the sample. The PCR primers are thus preferably chosen to amplify the unique target sequence and no other. Alternatively, the PCR primers may be selected to bind to sequences other than the target sequence where the amplification products can be subsequently distinguished (*e.g.* where the desired amplified sequence is different in size than other amplified sequences).

"Amplifying" or "amplification", which typically refer to an "exponential" increase in target nucleic acid, are used herein to describe both linear and exponential increases in the number of a select target sequence of nucleic acid.

The term "antisense orientation" refers to the orientation of nucleic acid sequence from a structural gene that is inserted in an expression cassette in an inverted manner with respect to its naturally occurring orientation. When the sequence is double stranded, the strand that is the template strand in the naturally occurring orientation becomes the coding strand, and vice versa.

The term "deletion" refers to a region of a nucleic acid which is not present in an organism, but which is present in another related organism. In the context of mycobacteria, a deletion refers, *e.g.*, to a region of nucleic acid which is not present in one strain of mycobacteria, but which is present in another related strain. For instance, an avirulent mycobacterial strain can have a deletion in its genome relative to the genome of a related virulent mycobacterial strain.

The term "deletion junction" refers to the region of a nucleic acid spanning the insertion point of a deletion. Thus, where a region of a nucleic acid sequence is deleted (*i.e.* a deletion is present), the deletion junction spans the nucleotides that are immediately adjacent to the deletion. Conversely, where a region of a nucleic acid sequence is not

deleted (*i.e.* the deletion is absent), two deletion junctions are present, each spanning respectively one end of the deletion sequence and its flanking sequence.

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, such as a polynucleotide sequence of Figures **1**, **2**, or **3**, or may comprise a complete cDNA or gene sequence.

Generally, a reference sequence is at least 10 nucleotides in length, frequently at least 20 to 25 nucleotides in length, and often at least 50 nucleotides in length. Sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a segment of at least 10 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 10 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences.

Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48: 443 (1970); by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. (USA)* 85: 2444 (1988), or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (*i.e.*, resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected.

The term "sequence identity" means that two polynucleotide sequences are identical (*i.e.*, on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned

sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.*, A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The term "identical" in the context of two nucleic acid or polypeptide sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence.

The terms "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. The isolated nucleic acid probes of this invention do not contain materials normally associated with their *in situ* environment, in particular nuclear, cytosolic or membrane associated proteins or nucleic acids other than those nucleic acids intended to comprise the nucleic acid probe itself.

The term "marker" refers to a characteristic which distinguishes one class of cells or compositions from a second class of cells or compositions. For instance, the deletions and deletion junctions described herein can be used to distinguish between strains (*e.g.*, virulent and avirulent strains) of mycobacteria. While markers are indicators of associated features or properties, as used herein, markers may also be used for purposes other than indicating the associated feature or property. Thus, for example, a nucleic acid marker of virulence identifies a particular nucleic acid which may be used in a variety of contexts other than simply indicating virulence.

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompassing known analogues of natural nucleotides that can function in a similar manner as naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence includes the complementary sequence thereof.

The term "operably linked" refers to functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates transcription of RNA corresponding to the second sequence.

The term "peptide" or "polypeptide" refers to an amino acid polymer which is encoded by a nucleic acid. The peptide or polypeptide may include naturally occurring or modified amino acids.

The terms "probe" or "nucleic acid probe" refer to a molecule that binds to a specific sequence or subsequence of a nucleic acid. A probe is preferably a nucleic acid which binds through complementary base pairing to the full sequence or to a subsequence of a target nucleic acid. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarily with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labelled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labelled such with, *e.g.*, biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the selected sequence or subsequence.

The term "labeled nucleic acid probe" refers to a nucleic acid probe that is bound, either covalently, through a linker, or through ionic, van der Waals or hydrogen "bonds" to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe.

The term "recombinant" when used with reference to a cell indicates that the cell replicates or expresses a nucleic acid, or expresses a peptide or protein encoded by DNA whose origin is exogenous to the cell. Recombinant cells can express genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also express genes found in the native form of the cell wherein the genes are re-introduced into the cell by artificial means.

The term "sample" refers to a material with which bacteria may be associated. Frequently the sample will be a "clinical sample" which is a sample derived from a patient. Such samples include, but are not limited to, sputum, blood, blood cells (*e.g.*, white cells), tissue or fine needle biopsy samples, urine, peritoneal fluid, and pleural fluid, or cells therefrom. It will be recognized that the term "sample" also includes supernatant from eukaryotic cell cultures (which may contain free bacteria), cells from cell or tissue culture, and other media in which it may be desirable to detect mycobacteria (*e.g.*, food and water).

The term "subsequence" in the context of a particular nucleic acid sequence refers to a region of the nucleic acid equal to or smaller than the specified nucleic acid.

The term "substantial identity" or "substantial similarity" indicates that a nucleic acid or polypeptide comprises a sequence that has at least 90% sequence identity to a reference sequence, or preferably 95%, or more preferably 98% sequence identity to the

reference sequence, over a comparison window of at least about 10 to about 100 nucleotides or amino acid residues. An indication that two polypeptide sequences are substantially identical is that one protein is immunologically reactive with antibodies raised against the second protein. An indication that two nucleic acid sequences are substantially identical is that the polypeptides which the first nucleic acids encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence-dependent and will be different with different environmental parameters. Generally, stringent conditions are selected to be about 5°C to 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0.2 molar at pH 7 and the temperature is at least about 60°C.

The term "uninterrupted reading frame" or "open reading frame" refers to a DNA sequence (*e.g.*, cDNA) lacking a stop codon or other intervening, untranslated sequence. An intact open reading frame refers to a full length uninterrupted reading frame or minor variations thereof.

The term "virulent" in the context of mycobacteria refers to a bacterium or strain of bacteria that replicates within a host cell or animal at a rate that is detrimental to the cell or animal within its host range. More particularly virulent mycobacteria persist longer in a host than avirulent mycobacteria. Virulent mycobacteria are typically disease producing and infection leads to various disease states including fulminant disease in the lung, disseminated systemic milliary tuberculosis, tuberculosis meningitis, and tuberculosis abscesses of various tissues. Infection by virulent mycobacteria often results in death of the host organism. Typically, infection of guinea pigs is used as an assay for mycobacterial virulence. In contrast, the term "avirulent" refers to a bacterium or strain of bacteria that either does not replicate within a host cell or animal within its host range, or replicates at a rate that is not significantly detrimental to the cell or animal.

The term BCG-like avirulence, as used herein refers to an attenuated virulence brought about by one of the deletions of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the complete sequence listing of the BCG deletion region 1 including flanking sequences. The deletion, designated BCGΔ1, is located between nucleotide 2327 and nucleotide 11126.

Figure 2 shows the complete sequence listing of the BCG deletion region 2 including flanking sequences. The deletion, designated BCGΔ2, is located between nucleotide 3382 and nucleotide 14071.

Figure 3 shows the complete sequence listing of the BCG deletion region 3 including flanking sequences. The deletion, designated BCGΔ3, is located between nucleotide 1406 and nucleotide 10673. "N" represents "A", "C", "G", or "T".

Figure 4 shows a map of the deletion sequence BCGΔ1. This map identifies the various open reading frames (ORFs) and indicates their location within the deletion sequence. Ribozome binding sites and homologies to the predicted encoded proteins are shown.

Figure 5 shows a map of the deletion sequence BCGΔ2. This map identifies the various open reading frames (ORFs) and indicates their location within the deletion sequence. Ribozomal binding sites and homologies to the predicted encoded proteins are shown.

Figure 6 shows a map of the deletion sequence BCGΔ3. This map identifies the various open reading frames (ORFs) and indicates their location within the deletion sequence. Ribozome binding sites and homologies to the predicted encoded proteins are shown. The sequence of a small region, estimated to be much less than 200 bp and located close to 9400 bp in Figure 3, remains to be determined. Therefore, the base pair coordinates given in the region 3 map 3' to the 9kb marker are approximations. The precise sequence determination of this region is likely to effect the length of open reading frames 3H and 3L.

Figure 7 illustrates the deletion junction regions of BCGΔ1, BCGΔ2, and BCGΔ3. The "terminal" deletion junction regions formed by the flanking sequences and the terminal regions of the deletion sequences are identified as BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, and BCGΔ3a, and BCGΔ3b. When the deletion is present (the deletion sequences

are missing) the respective "a" and "b" sequences will be juxtaposed, thereby forming deletion "spanning" junction sequences designated BCGΔ1ab, BCGΔ2ab, and BCGΔ3ab, respectively.

Figure 8 shows EcoRI and BamHI restricted chromosomal DNAs from *Mycobacterium bovis*, BCG Connaught, and *Mycobacterium tuberculosis* strains H37Ra, H37Rv, and Erdman probed with ^{32}P labeled BCG subtracted probe.

DETAILED DESCRIPTION

This invention reflects the discovery of genetic deletions in mycobacteria that result in an avirulent genotype such as is exhibited by the bacille Calmette-Guérin (BCG) mycobacterium. The original Pasteur bacille Calmette-Guérin (BCG) strain was developed by multiple (230 times) serial passages in liquid culture. BCG has never been shown to revert to virulence in animals indicating that the attenuating mutations in BCG are stable deletions and/or multiple mutations that cannot revert. The mutations that arose during serial passage of the original BCG strain were not previously known. Recent efforts to genetically complement BCG virulence with genomic libraries of virulent tubercle bacilli were unsuccessful, again suggesting that multiple unlinked mutations are responsible for the attenuation of BCG virulence.

The genetic deletions leading to the avirulent phenotype of BCG were identified by genomic subtractions between Connaught strain of BCG and MBV/MTB. The subtracted probe resulting from the genomic subtraction between BCG and the H37 Rv strain of *M. tuberculosis* was subsequently used to identify and clone three regions from a cosmid library of *Mycobacterium bovis* genomic DNA. Southern blot mapping and DNA sequence comparisons between BCG and *M. bovis* showed that three regions, designated regions 1-3, contained DNA segments of approximately 9 kb, 11 kb and 9 kb respectively, which are deleted in the Connaught strain of BCG. Precise deletion junctions were identified for each region by comparisons of BCG and corresponding virulent MBV sequences. The respective deletions, designated BCGΔ1, BCGΔ2 and BCGΔ3 are illustrated in Figures 1-3.

One of skill in the art will appreciate that the deletions encompassed by BCGΔ1, BCGΔ2 and BCGΔ3 may be utilized in a variety of contexts. For example, the deletions may be utilized to distinguish between avirulent and virulent strains of

mycobacteria thereby providing early detection of patients at risk for tuberculosis. This is of particular importance where mycobacteria are identified in a sample from a patient that has been previously vaccinated with BCG. In this context it may be critical to determine whether mycobacteria identified in a biological sample from such a patient are pathogenic.

In another embodiment, the preparation of mycobacteria containing the deletions of the present invention may provide superior vaccines to BCG which has long been known to have marginal efficacy. Thus, for example, a *Mycobacterium tuberculosis* may contain a full BCGΔ1 deletion or a smaller deletion within BCGΔ1 (*e.g.* one or more open reading frames) rendering it avirulent. An avirulent MTB will provide a more efficient vaccine because it is antigenically more similar to MTB than is BCG. Moreover, an MTB rendered avirulent by the production of smaller deletions within the deletion regions identified in this invention will present more antigenic determinants.

Since the loss of virulence is due to the loss of gene products expressed by the nucleic acid sequences comprising the deletion regions, the BCGΔ1, BCGΔ2 and BCGΔ3 deletion sequences and proteins encoded within these deletion sequences provide suitable targets for drug screening. Thus, the use of deleted sequences as targets to screen for drugs that inhibit or interfere with transcription, translation, or post-translational processing of proteins encoded by the deletion sequences, or with the deletion encoded polypeptides themselves, provides an assay for anti-mycobacterial agents. In particular, the use of reporter genes such as firefly luciferase (FFlux), ß-galactosidase (BGal), and the like, under the control of promoters present in the deletion sequence provide a rapid assay for drugs regulating activity originating in this region. Conversely, since the protein products of the deletion sequences are presumably expressed in virulent mycobacterial species, proteins expressed by deletion sequences may make good antigens for antimycobacterial vaccines.

Finally, as the viability of BCG demonstrates, deletion regions BCGΔ1, BCGΔ2 and BCGΔ3 are not required for mycobacterial growth and reproduction. Thus, these deletion regions provide good insertion points for the expression of heterologous DNA. The heterologous DNA sequences may be under the control of endogenous inducible or constitutive promoters typically found in the deletion sequences, or alternatively, they may be under the control of introduced promoters, either constitutive or inducible, exogenous to mycobacteria.

I. Detection of Deletions

As indicated above, the deletions identified in the present invention provide useful markers for the identification of an avirulent (or conversely a virulent) mycobacterial phenotype. Specifically, determination of avirulence simply requires the detection of the presence or absence of the deletion (either BCGΔ1, BCGΔ2, or BCGΔ3, or deletions within these regions). Where the deletion is present in the bacterial DNA, the bacterium expresses a BCG-like avirulent phenotype. Conversely, where the deletion is absent in the bacterial DNA, the bacterium does not express a BCG-like avirulence. While this may indicate that the bacterium is virulent, one of skill will appreciate that the bacterium may still be avirulent due to the presence of other mutations or deletions. Nevertheless, screening for the presence of the deletion provides a means of detecting a BCG-like avirulent mycobacterium.

Means of detecting deletions are well known to those of skill in the art. Generally, the deletions may be detected either by detecting the presence or absence of deletion junctions, or, alternatively, by detecting the presence or absence of the sequences contained within the deletion (deletion sequences). Where a nucleic acid sequence is deleted (*i.e.*, a deletion is present), the sequences that previously flanked the deleted sequence are juxtaposed, thereby forming a new deletion junction that spans the deletion. Detection of the presence of such a "spanning" deletion junction indicates the presence of the deletion and thus the avirulent phenotype.

Conversely, where the nucleic acid sequence is not deleted (the deletion is not present) the spanning junction sequence will be absent (See, *e.g.* Figure 7). The "terminal" deletion junction sequences flanking each endpoint of the deletion region are present and detection of these terminal deletion junctions indicates the absence of a deletion. Spanning deletion junction regions and terminal deletion junctions suitable for detecting the deletions of the present invention are illustrated in Figure 7 and in Table 1.

Table 1. Nucleic acid sequences comprising deletion junctions. The symbol "|" indicates the insertion point of the deletion sequence. Deletion sequence bases are represented in lower case letters.

Junction	Nucleotide Sequence	Seq. ID
BCGΔ1a	CTGGTCGACGATTGGCACAT|gcagccgtgggtgccgccgg	1

BCGΔ1b	gtgtcttcatcggcttccac|CCAGCCGCCCGGATCCAGCA	2
BCGΔ2a	CAACTCCACGGCGACCACCC|gcgcccccgctcgcactaga	3
BCGΔ2b	gcccacccggtcgagcaccc|CGATGATCTTCTGTTTGACC	4
BCGΔ3a	CACCTCGACCACGGCCAACC|gtggacctgtgagatacact	5
BCGΔ3b	tcagcagtccacggccaacc|CCGCACCAACACCTTCCACC	6
BCGΔ1ab	CTGGTCGACGATTGGCACAT|CCAGCCGCCCGGATCCAGCA	7
BCGΔ2ab	CAACTCCACGGCGACCACCC|CGATGATCTTCTGTTTGACC	8
BCGΔ3ab	CACCTCGACCACGGCCAACC|CCGCACCAACACCTTCCACC	9

Where a deletion is detected by determining the presence or absence of sequences contained within the deletion (deletion sequences), the absence of deletion sequences indicates the presence of a deletion and thus an avirulent phenotype. Conversely, the presence of deletion sequences indicates the absence of a deletion. Deletion sequences that provide suitable targets for detecting the deletions of the present invention are provided in Figures 1, 2 and 3.

A) Isolation of DNA for Detection of Mycobacterium Genomic Deletions

In a preferred embodiment, DNA is obtained from mycobacteria. As used herein, the term "mycobacteria" refers to any bacteria of the family *Mycobacteriaceae* (order *Actinomycetales*) and includes, but is not limited to, *Mycobacterium tuberculosis*, *Mycobacterium avium complex*, *Mycobacterium kansasii*, *Mycobacterium scrofulaceum*, *Mycobacterium bovis and Mycobacterium leprae*. These species and groups and others are described in Baron, S., ed. *Medical Microbiology*, 3rd Ed. (1991) Churchill Livingstone, New York, which is incorporated herein by reference.

The identification of deletions using a DNA marker requires that the DNA sequence be accessible to the particular probes used or to the components of the amplification system if the DNA sequence is to be amplified. In general, this accessibility is ensured by isolating the nucleic acids from the sample.

A variety of techniques for extracting nucleic acids from biological samples are known in the art. For example, see those described by Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New

York, (1985), by Han, *et al. Biochemistry*, 26: 1617-1625 (1987) and by Du, *et al. Bio/Technology*, 10: 176-181 (1992), which are incorporated herein by reference.

Alternatively, if the sample is readily disruptable, the nucleic acid need not be purified prior to amplification by the PCR technique, *i.e.*, if the sample is comprised of cells, particularly peripheral blood lymphocytes or monocytes, lysis and dispersion of the intracellular components may be accomplished merely by suspending the cells in hypotonic buffer or boiling them in a low concentration of alkali (*i.e.* 10 mM NaOH).

In a preferred embodiment, DNA is extracted from mycobacteria as described in Example 1.

B) Detection of Deletions Using Hybridization Probes

In one embodiment the avirulence deletions are detected by contacting DNA obtained from the mycobacterium with a probe that specifically binds an entire deletion junction region or a subsequence of that region and does not specifically bind to any other DNA sequences in the sample. Alternatively, a probe that specifically binds the entire deleted region or subsequence of that region and does not specifically bind to any other sequences in the sample is also suitable. While such probes may be proteins, oligonucleotide probes are preferred. Typically, the sequence of the oligonucleotide probe is chosen to be complementary to a select subsequence unique to the deletion junction or the deletion sequence, whose presence or absence is to be detected. Under stringent conditions the probe will hybridize with the select subsequence forming a stable duplex.

The probe is typically labeled. Detection of the label in association with the target DNA indicates either the presence or absence of the deletion. The probe may be used to detect the deletion junction or deletion sequences directly in a DNA sample without amplification of the deletion subsequences. In one embodiment, unamplified DNA sequences are probed using a Southern blot. The DNA of the sample is immobilized, on a solid substrate, typically a nitrocellulose filter or a nylon membrane. The substrate-bound DNA is then hybridized with the labeled probe under stringent conditions and non-specifically hybridized probe is washed away. Labeled probe detected in association with the immobilized mycobacterial sequences (*e.g.* bound to the substrate) indicates the presence of deletion sequences (*e.g.* BCGΔ1, BCGΔ2, or BCGΔ3) and therefore the absence of the deletion. Means for detecting specific DNA sequences are well known to those of skill in

the art. Protocols for Southern blots as well as other detection methods are provided in Maniatis, *et al. Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, NY (1982), which is incorporated herein by reference.

In another embodiment, the mycobacterial DNA subsequences are themselves labeled. They are then hybridized, under stringent conditions, with a probe immobilized on a solid substrate. Detection of the label in association with the immobilized probe indicates the presence or absence of the deletion.

In a preferred embodiment, the deletion junction sequences or subsequences or the deletion sequences or subsequences may be amplified by a variety of DNA amplification techniques (for example via cloning, polymerase chain reaction, ligase chain reaction, transcription amplification, *etc.*) prior to detection using a probe. Because the copy number of mycobacterial sequences bearing the virulence-attenuating deletions is low, the use of unamplified mycobacterial DNA results in an assay of low sensitivity. Amplification of mycobacterial DNA increases sensitivity of the assay by providing more copies of possible target subsequences. In addition, by using labeled primers in the amplification process, the mycobacterial DNA sequences are labeled as they are amplified.

C) Selection of Probes for Detection of the Deletion Junction Sequences or the Deletion Sequences

Full length sequences are provided for the deletions BCGΔ1, BCGΔ2, and BCGΔ3 in Figures 1, 2 and 3 respectively. Using these sequence listings, one of skill in the art may easily determine appropriate probes or primers for the detection of the presence or absence of the deletion junctions or the deletion sequences. Generally speaking, a probe will be selected that hybridizes to the target junction sequences or deletion sequences, but not to other mycobacterial nucleic acid sequences under stringent conditions. The design of hybridization probes is well known in the art. See, for example, Sambrook *et al., Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989), which is incorporated herein by reference.

In a preferred embodiment, the probe is an oligonucleotide sequence complementary to a subsequence comprising a deletion junction (*e.g.* BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, and BCGΔ3ab) or a

sequence complementary to a subsequence of a deletion sequence (*e.g.* BCGΔ1, BCGΔ2, and BCGΔ3). The probe preferably has destabilizing mismatches with subsequences from other regions of the mycobacterial genome.

The exact length of the probe depends on many factors including the length of conserved regions around the deletions, the degree of sequence specificity desired, and the amount of internal complementarity within the probe. Such probes are preferably 17 to 25 bases in length. One of skill will recognize that longer probes specifically hybridize at higher temperatures. Generally, stringent conditions are selected to be about 5°C to 20°C, more preferably about 10°C, lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. Under stringent conditions, the probe will specifically hybridize to a nucleic acid sequence from an avirulent mycobacterium such as BCG, but not to a nucleic acid sequence from a virulent mycobacterium such as MTB or MBV. Alternatively, Under stringent conditions, the probe will specifically hybridize to a nucleic acid sequence from a avirulent mycobacterium such as MTB or MBV, but not to a nucleic acid sequence from an avirulent mycobacterium such as BCG.

Oligonucleotide probes can be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences and direct chemical synthesis by a method such as the phosphotriester method of Narang *et al. Meth. Enzymol*, 68: 90-99 (1979); the phosphodiester method of Brown *et al.*, *Meth. Enzymol.* 68:109-151 (1979); the diethylphosphoramidite method of Beaucage *et al.*, *Tetra. Lett.*, 22: 1859-1862 (1981); and the solid support method of U.S. Patent No. 4,458,066.

Probe detectability may be increased by the attachment of a label. As used herein, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads™), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*) beads.

Methods for attaching labels to probes, primers, and antibodies are well known to those of skill in the art. For example, the probe can be labeled at the 5'-end with ^{32}P by incubating the probe with ^{32}P-ATP and polynucleotide kinase (see Perbal, *A*

Practical Guide to Molecular Cloning, 2nd ed. John Wiley, N.Y. (1988)). Other labels may be joined to the probe directly or through linkers. They may be located at the ends of the probe or internally. Methods of attaching labels may be found in Connell, *et al.*, *Bio/Techniques* 5: 342 (1987), U.S. Patent Nos. 4,914,210, 4,391,904 and 4,962,029, which are incorporated herein by reference. In addition, kits for labelling oligonucleotides are widely available. See, for example, Boehringer Mannheim Biochemicals (Indianapolis, IN) for "Genius" labeling kits based on dioxigenin technology and Clonetech (South San Francisco, CA) for a variety of direct and indirect oligonucleotide labeling reagents.

D) Detection of Deletions Conferring Avirulence Through Amplification of Unique Subsequences

Deletions are particularly amenable to detection without the use of a hybridization probe. In a preferred embodiment, subsequences are amplified that include a deletion junction. The amplified deletion junction may be a "spanning" deletion junction in which case where the deletion is present (*i.e.* the deletion sequences are absent), the amplification product is a specific DNA incorporating the deletion junction sequence spanning the deletion (*e.g.* incorporating flanking sequences from both sides of the deleted sequence). Where the deletion is absent (*i.e.* deletion sequences are present) and primers are selected so that there are no priming sites within the deletion sequences, amplification is non-existent or alternatively provides a complex mixture of non-specifically amplified fragments. Alternatively, amplification primers may be selected that specifically hybridize to deletion sequences, as long as they are selected to amplify sequences that are distinguishable from the sequence amplified when the deletion is present.

Alternatively, the amplification product may be subsequence of a "terminal" deletion junction in which case absence of the deletion (*i.e.* the deletion sequences are present) will result in the amplification of the specifically targeted nucleic acid. Conversely, where the deletions are present (*i.e.* the deletion sequences are absent) there will be no specific amplification of a terminal deletion junction.

Amplification products may be separated by size for characterization. Size separation may be accomplished by a variety of means known to those of skill in the art.

These methods include, but are not limited to electrophoresis, density gradient centrifugation, liquid chromatography, and capillary electrophoresis. In a preferred embodiment, the fragments are separated by agarose gel electrophoresis. The bands are then stained with a marker to visualize them such as ethidium bromide and the gel is visualized, *e.g.*, using ultraviolet light.

As described above, an agarose gel typically shows 1 band if the deletion is present, reflecting amplification of the deletion-spanning sequence. Where the deletion is absent, amplification results in either no bands, where there are no sequences within the deletion to which the amplification primers may hybridize, or a smear where there is non-specific amplification, or a series of discrete bands distinguishable from the band representing the deletion-spanning sequence where primers are chosen that hybridize to deletion sequences.

E) Selection of Primers for Amplification of Avirulence Deletions

Amplification of deletion junction sequences or subsequences or deletion sequences or subsequences may be accomplished by methods well known in the art, which include, but are not limited to polymerase chain reaction (PCR) (Innis, *et al.*, *PCR Protocols. A guide to Methods and Application.* Academic Press, Inc. San Diego, (1990), which is incorporated herein by reference), ligase chain reaction (LCR) (see Wu and Wallace, *Genomics*, 4: 560 (1989), Landegren, *et al.*, *Science*, 241: 1077 (1988) and Barringer, *et al.*, *Gene*, 89: 117 (1990), which are incorporated herein by reference), transcription amplification (see Kwoh, *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 86: 1173 (1989) which is incorporated herein by reference), and self-sustained sequence replication (see Guatelli, *et al.*, *Proc. Nat. Acad. Sci. (U.S.A.)*, 87: 1874 (1990) which is incorporated herein by reference), each of which provides sufficient amplification so that the target sequence can be detected by nucleic acid hybridization to a probe or by electrophoretic separation. Alternatively, methods that amplify the hybridization probe to detectable levels can be used, such as Qβ-replicase amplification. See, for example, Kramer, *et al. Nature*, 339: 401 (1989), Lizardi, *et al. Bio/Technology*, 6: 1197 (1988), and Lomell, *et al.*, *Clin. Chem.* 35: 1826 (1989) which are incorporated herein by reference.

In a preferred embodiment, amplification is by polymerase chain reaction using a pair of primers that flank and thereby amplify a selected deletion junction subsequence. Selection of primers is readily apparent to one of skill in the art using the sequence listings of the present invention. For example, a pair of PCR primers 5'-TCGACGATTGGCACAT-3' (T_m=55°C) and 5'-TCCCTCCCTGTATTTGTAT-3' (T_m=56°C) will amplify a 469 base pair sequence including the BCGΔ1a deletion junction, while 5'-CGTTCTTCGGAGGTTTC-3' (T_m=56°C) and 5'-GGCGGCTGGGTGGA-3' (T_m=60°C) will amplify a 471 base pair sequence including the BCGΔ1b deletion junction.

F) Detection of Deletions through Detection of Expression Products of Deletion Sequences

In addition to the detection of deletions by the detection of either the deletion junction sequences or the deletion sequences, one may detect the absence of the deletion by detecting the expression products of the deletion sequences. Thus, for example, where the deletion sequences express a protein, the presence of that protein indicates the absence of the deletion and thus is indicative of a virulent (non BCG-like) phenotype. Such proteins are referred to herein as "deletion polypeptides".

Means of determining proteins expressed by particular nucleic acid sequences are well known to those of skill in the art. Typically this involves determining the longest open reading frame. This may be aided by the identification of initiation sites (*e.g.* ribozome binding sites). The protein encoded by the largest open reading frame is determined using codon preferences for the specific organism from which the nucleic acid is obtained. The polypeptide sequence listing may then be compared against a sequence database, *e.g.* GenBank, to determine other sequences sharing substantial sequence identity with the calculated sequence. The expression of the protein may be verified by isolating and then sequencing proteins having the predicted length and charge characteristics.

Once deletion polypeptides are identified they may be detected by routine methods well known to those of skill in the art. Typically this involves isolating and then detecting the polypeptide. The polypeptide may be isolated by a number of means well known to those of skill in the art. This includes typical methods of protein

purification such as high performance liquid chromatography (HPLC), electrophoresis, capillary electrophoresis, hyperdiffusion chromatography, thin layer chromatography, and the like. Methods of purifying and detecting proteins are well known to those of skill in the art (see, *e.g.*, *Methods in Enzymology Vol. 182: Guide to Protein Purification*, M. Deutscher, ed. Vol. 182 (1990), which is incorporated herein by reference).

Alternatively, deletion polypeptides sequences may be detected using immunoassays utilizing antibodies specific for the deletion polypeptides. The production of such antibodies and their use in immunoassays is detailed below.

G) Antibodies to Deletion Polypeptides

Antibodies can be raised to the polypeptides encoded by the nucleic acids corresponding to the open reading frames present in the deletion regions of the present invention (deletion polypeptides). As used herein "antibodies" include immunoglobulin or a population of immunoglobins which specifically bind to an antigen. Thus an antibody may be monoclonal or polyclonal including individual, allelic, strain, or species variants, and fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms. Additionally, antibodies can be raised to these polypeptides in either their native configurations or in non-native configurations. Anti-idiotypic antibodies may also be used.

1) Antibody Production

A number of immunogens may be used to produce antibodies specifically reactive with deletion polypeptides. Recombinant polypeptides are the preferred immunogen for the production of monoclonal or polyclonal antibodies. Naturally occurring polypeptides may also be used either in pure or impure form. Synthetic peptides made using sequences described herein may also used as immunogens for the production of antibodies.

Recombinant polypeptides are expressed in eukaryotic or prokaryotic cells and purified using standard techniques. The polypeptide is injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies may be generated for subsequent use in immunoassays to measure the presence and quantity of the polypeptide.

Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen, preferably a purified deletion polypeptide is mixed with an adjuvant and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired. *See, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY, which are incorporated herein by reference.

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Description of techniques for preparing such monoclonal antibodies may be found in, *e.g.*, Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane (1988) *Antibodies: A Laboratory Manual* CSH Press; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and particularly in Kohler and Milstein (1975) *Nature* 256: 495-497, which discusses one method of generating monoclonal antibodies.

Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are then fused with myeloma cells (*See*, Kohler and Milstein (1976) *Eur. J. Immunol.* 6: 511-519, incorporated herein by reference). The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*.

Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells is enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse *et al.* (1989) *Science* 246: 1275-1281. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B

cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Other suitable techniques involve selection of libraries of antibodies in phage or similar vectors. See, Huse *et al. Science* 246: 1275-1281 (1989); and Ward, *et al. Nature* 341: 544-546 (1989). The polypeptides and antibodies of the present invention are used with or without modification, including chimeric antibodies. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents, teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced. See, Cabilly, U.S. Patent No. 4,816,567; and Queen *et al. Proc. Nat'l Acad. Sci. USA* 86: 10029-10033 (1989).

Antibodies, including binding fragments and single chain versions, against predetermined fragments of deletion polypeptides can be raised by immunization of animals with conjugates of the fragments with carrier proteins as described above. Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies can be screened for binding to normal or defective polypeptides, or screened for agonistic or antagonistic activity, *e.g.*, mediated through a receptor. These monoclonal antibodies will usually bind with at least a K_D of about 1 mM, more usually at least about 300 μM, and most preferably at least about 0.1 μM or better.

The antibodies of this invention can also be used for affinity chromatography in isolating deletion polypeptides. Columns can be prepared where the antibodies are linked to a solid support, *e.g.*, particles, such as agarose, Sephadex, or the like, where a bacterial lysate, or recombinant cell lysate is passed through the column, washed, and treated with increasing concentrations of a mild denaturant, whereby purified deletion polypeptides are released.

The antibodies can be used to screen expression libraries for particular expression products. Usually the antibodies in such a procedure will be labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

In a preferred embodiment, antibodies to deletion polypeptides are used for the identification of cell populations expressing the polypeptides. By assaying the expression products of cells expressing the polypeptides it is possible to diagnose bacterial infections.

Antibodies raised against each polypeptide are useful to raise anti-idiotypic antibodies. These will be useful in detecting or diagnosing various immunological conditions related to the presence of the respective antigens.

2) Immunoassays

A particular deletion polypeptide can be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures in general, see Stites and Terr (eds.) 1991 *Basic and Clinical Immunology* (7th ed.). Moreover, the immunoassays of the present invention can be performed in any of several configurations, *e.g.*, those reviewed in Maggio (ed.) (1980) *Enzyme Immunoassay* CRC Press, Boca Raton, Florida; Tijan (1985) "Practice and Theory of Enzyme Immunoassays," *Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers B.V., Amsterdam; and Harlow and Lane *Antibodies, A Laboratory Manual, supra*, each of which is incorporated herein by reference. See also Chan (ed.) (1987) *Immunoassay: A Practical Guide* Academic Press, Orlando, FL; Price and Newman (eds.) (1991) *Principles and Practice of Immunoassays* Stockton Press, NY; and Ngo (ed.) (1988) *Non-isotopic Immunoassays* Plenum Press, NY.

Immunoassays for measurement of deletion polypeptides can be performed by a variety of methods known to those skilled in the art. In brief, immunoassays to measure the protein can be, *e.g.*, competitive or noncompetitive binding assays. In competitive binding assays, the sample to be analyzed competes with a labeled analyte for specific binding sites on a capture agent bound to a solid surface. Preferably the capture agent is an antibody specifically reactive with a deletion polypeptide produced as described above. The concentration of labeled analyte bound to the capture agent is inversely proportional to the amount of free analyte present in the sample.

In a competitive binding immunoassay, the deletion polypeptide present in the sample competes with labelled protein for binding to a specific binding agent, for example, an antibody specifically reactive with a particular deletion polypeptide. The binding agent is, *e.g.*, bound to a solid surface to produce separation of bound labelled polypeptide from the unbound labelled polypeptide. Alternately, the competitive binding assay may be conducted in liquid phase and any of a variety of techniques known in the art may be used to separate the bound labelled protein from the unbound labelled protein. Following separation, the amount of bound labeled protein is determined. The amount of polypeptide present in the sample is inversely proportional to the amount of labelled polypeptide binding.

Alternatively, a homogenous immunoassay may be performed in which a separation step is not needed. In these immunoassays, the label on the protein is altered by the binding of the protein to its specific binding agent. This alteration in the labelled protein results in a decrease or increase in the signal emitted by label, so that measurement of the label at the end of the immunoassay allows for detection or quantitation of the polypeptide.

Deletion polypeptides may also be detected by a variety of noncompetitive immunoassay methods. For example, a two-site, solid phase sandwich immunoassay may be used. In this type of assay, a binding agent for the protein, for example an antibody, is attached to a solid support. A second protein binding agent, which is also an antibody, and which binds the protein at a different site, is labelled. After binding at both sites on the protein, the unbound labelled binding agent is removed and the labelled binding agent bound to the solid phase is measured. The amount of labelled binding agent bound is directly proportional to the amount of polypeptide in the sample.

Western blot analysis can be used to determine the presence of a deletion polypeptide in a sample. Electrophoresis is carried out, for example, on a bacterial sample suspected of containing the deletion polypeptide. Following electrophoresis to separate the proteins, and transfer of the proteins to a suitable solid support such as a nitrocellulose filter, the solid support is incubated with an antibody reactive with the protein. This antibody is labelled, or alternatively may be it is detected by subsequent incubation with a second labelled antibody that binds the primary antibody.

The immunoassay formats described above employ labelled assay components. The label can be in a variety of forms as described above. The choice of label depends on sensitivity required, ease of conjugation with the compound, stability requirements, and available instrumentation. For a review of various labelling or signal producing systems which may be used, see U.S. Patent No. 4,391,904, which is incorporated herein by reference.

Antibodies reactive with a particular protein can also be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures applicable to the measurement of antibodies by immunoassay techniques, see Stites and Terr (eds.) *Basic and Clinical Immunology* (7th ed.) *supra*; Maggio (ed.) *Enzyme Immunoassay*, *supra*; and Harlow and Lane *Antibodies, A Laboratory Manual*, *supra*.

In brief, immunoassays to measure antisera reactive with polypeptides include competitive and noncompetitive binding assays. In competitive binding assays, the sample analyte competes with a labeled analyte for specific binding sites on a capture agent bound to a solid surface. Preferably the capture agent is a purified recombinant deletion polypeptide as described above. Other sources of polypeptides, including isolated or partially purified naturally occurring protein, can also be used. Noncompetitive assays are typically sandwich assays, in which the sample analyte is bound between two analyte-specific binding reagents. One of the binding agents is used as a capture agent and is bound to a solid surface. The second binding agent is labelled and is used to measure or detect the resultant complex by visual or instrument means. A number of combinations of capture agent and labelled binding agent can be used. A variety of different immunoassay formats, separation techniques and labels can be also be used similar to those described above for the measurement of deletion polypeptides.

II. Preparation of Deletion-Containing Mycobacteria

Mycobacteria containing specific deletions may be prepared by using methods of homologous recombination well known to those of skill in the art. In brief, homologous recombination is a natural cellular process which results in the scission of two nucleic acid molecules having identical or substantially similar (*i.e.* "homologous") sequences, and the ligation of the two molecules such that one region of each initially

present molecule is now ligated to a region of the other initially present molecule (Sedivy, *Bio/Technol.*, 6: 1192-1196 (1988).

Homologous recombination is exploited by a number of various methods of "gene targeting" well known to those of skill in the art. (*see, for example*, Mansour *et al. Nature*, 336: 348-352 (1988); Capecchi *Trends Genet.* 5: 70-76 (1989); Capecchi *Science* 244: 1288-1292 (1989); Capecchi *et al.* pages 45-52 In: *Current Communications in Molecular Biology*, Capecchi, M.R. (ed.), Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989); Frohman *et al. Cell* 56: 145-147 (1989)). Some approaches focus on increasing the frequency of recombination between two DNA molecules by treating the introduced DNA with agents which stimulate recombination (*e.g.* trimethylpsoralen, UV light, *etc.*), however, most approaches utilize various combinations of selectable markers to facilitate isolation of the transformed cells.

One such selection method is termed positive/negative selection (PNS) (Thomas and Cappechi *Cell* 51: 503-512 (1987)). This method involves the use of two selectable markers: one a positive selection marker such as the bacterial gene for neomycin resistance (*neo*^{r}); the other a negative selection marker such as the herpes virus thymidine kinase (*tk*) gene. *Neo*^{r} confers resistance to the drug G-418, while herpes *tk* renders cells sensitive to the nucleoside analog gangcyclovir (GANC) or 1-(2-deoxy-2-fluoro-b-d-arabinofuranosyl)-5-iodouracil (FIAU). The DNA encoding the positive selection marker in the transgene (*e.g.* *neo*^{R}) is generally linked to an expression regulation sequence that allows for its independent transcription in mycobacteria. It is flanked by first and second sequence portions of at least a part of the deletion or deletion flanking sequences.

These first and second sequence portions target the transgene to a specific nucleotide sequence. A second independent expression unit capable of producing the expression product for a negative selection marker, *e.g.* for herpes virus tk is positioned adjacent to or in close proximity to the distal end of the first or second portions of the first DNA sequence. Upon transfection, some of the mycobacteria incorporate the transgene by random integration, others by homologous recombination between the endogenous allele and sequences in the transgene. As a result, one copy of the targeted nucleic acid is disrupted by homologous recombination with the-transgene with simultaneous loss of the sequence encoding herpes tk gene. Random integrants, which

occur via the ends of the transgene, contain herpes tk and remain sensitive to GANC or FIAU. Therefore, selection, either sequentially or simultaneously with G418 and GANC enriches for transfected mycobacteria containing the transgene integrated into the genome by homologous recombination.

Methods of homologous recombination in mycobacteria are described in greater detail by Ganjam *et al. Proc. Natl. Acad. Sci. USA*, 88: 5433-5437 (1991) and Aldovini *et al.*, *J. Bacteriol.*, 175: 7282-7289 (1993) which are incorporated herein by reference.

III. Screening for Drug Susceptibility/Therapeutics

The expression products of the open reading frames in the BCGΔ1, BCGΔ2, and BCGΔ3 deletions of the present invention are targets for anti-mycobacterial drugs. To determine particularly suitable drug targets, open reading frames and surrounding expression control sequences are introduced into avirulent strains of mycobacteria, alone or in combination with other open reading frame regions to determine which regions are critical for virulence. Once particular genes are identified as critical for virulence, anti-mycobacterial agents are designed to inhibit expression of the critical genes, or to attack the critical gene products. For instance, antibodies are generated against the critical gene products and used as prophylactic or therapeutic agents. Alternatively, small molecules can be screened for the ability to selectively inhibit expression of the critical gene products, *e.g.*, using recombinant expression systems which include the gene's endogenous promoter. These small molecules are then used as therapeutics, or prophylactic agents to inhibit mycobacterial virulence.

In another embodiment, anti-mycobacterial agents which render a virulent mycobacterium avirulent can be operably linked to expression control sequences and used to transform a virulent mycobacterium. Such anti-mycobacterial agents inhibit the replication of a specified mycobacterium upon transcription or translation of the agent in the mycobacterium.

Such transformed mycobacteria are useful as vaccine components, and as components of immunological infectivity assays. For instance, an animal's blood can be monitored for the presence of anti-mycobacterial antibodies using the procedures described herein, using transformed avirulent mycobacterial components in various

immunological assays. Anti-mycobacterial agents useful in this invention include, without limitation, antisense genes, ribozymes, decoy genes, transdominant proteins and suicide genes.

An antisense nucleic acid is a nucleic acid that, upon expression, hybridizes to a particular mRNA molecule, to a transcriptional promoter or to the sense strand of a gene. By hybridizing, the antisense nucleic acid interferes with the transcription of a complementary DNA, the translation of an mRNA, or the function of a catalytic RNA. Antisense molecules useful in this invention include those that hybridize to gene transcripts in the region of the deletions of the invention, particularly deletion region 1.

A ribozyme is a catalytic RNA molecule that cleaves other RNA molecules having particular nucleic acid sequences. Ribozymes useful in this invention are those that cleave deletion gene transcripts. Examples include hairpin and hammerhead ribozymes.

A decoy nucleic acid is a nucleic acid having a sequence recognized by a regulatory DNA binding protein (*i.e.*, a transcription factor). Upon expression, the transcription factor binds to the decoy nucleic acid, rather than to its natural target in the genome. Useful decoy nucleic acid sequences include any sequence to which a transcription factor binds in the deletion regions of the present invention.

A transdominant protein is a protein whose phenotype, when supplied by transcomplementation, will overcome the effect of the native form of the protein. For instance, an avirulent mycobacterium can be rendered virulent by introducing transdominant proteins from deletion region 1.

A suicide gene produces a product which is cytotoxic. In the vectors of the present invention, a suicide gene is operably linked to an inducible expression control sequences which is stimulated upon infection of a cell by a mycobacterium.

IV. Use of Expressed "Deletion Proteins" in a Vaccine

The deletion polypeptides encoded by the open reading frames in BCGΔ1, BCGΔ2, and BCGΔ3 may be recombinantly expressed and used as components of immunological assays as described above or in vaccines. Expression of polypeptides

encoded by the open reading frames of the BCGΔ1, BCGΔ2, or BCGΔ3 deletions may be accomplished by means well known to those of skill in the art.

In brief, the expression of natural or synthetic nucleic acids encoding deletion polypeptides will typically be achieved by operably linking the DNA or cDNA to a promoter (which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of polynucleotide sequence encoding deletion polypeptides.

To obtain high level expression of a cloned gene, such as those polynucleotide sequences encoding deletion polypeptides, it is desirable to construct expression plasmids which contain, at the minimum, a promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. The expression vectors may also comprise generic expression cassettes containing at least one independent terminator sequence, sequences permitting replication of the plasmid in both eukaryotes and prokaryotes, *i.e.*, shuttle vectors, and selection markers for both prokaryotic and eukaryotic systems. For detailed techniques employed in the recombinant expression of deletion proteins *see*, for example, Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory (1989)), *Methods in Enzymology*, Vol. 152: *Guide to Molecular Cloning Techniques* (Berger and Kimmel (eds.), San Diego: Academic Press, Inc. (1987)), or *Current Protocols in Molecular Biology*, (Ausubel, *et al.* (eds.), Greene Publishing and Wiley-Interscience, New York (1987), all of which are incorporated herein by reference.

The expressed deletion polypeptides may be used in a variety of assays. For example, the deletion polypeptides can be used as reagents in immunoblot assays to test whether a patient was previously exposed to virulent mycobacteria (*i.e.*, to test whether the patient has antibodies to the deletion polypeptide). These assays have the advantage of discriminating between previous exposure to an avirulent mycobacterium (*e.g.*, one used in a vaccine) and exposure to a virulent mycobacterium. Thus, vaccinated individuals can be tested for antibodies to the virulent mycobacterium without regard to whether the patient has been vaccinated with an avirulent mycobacterium.

The deletion polypeptides can also be used as antigenic vaccine components to direct antibodies to elements which are critical for virulence. These polypeptides can be added to existing vaccines (*e.g.*, those based upon avirulent mycobacteria and which lack the deletion polypeptide) to supplement the range of antigenicity conferred by the vaccine, or they may be used apart from other mycobacterial antigens. The vaccines of the invention contain as an active ingredient an immunogenically effective amount of a deletion polypeptide or of a recombinant vector which includes the deletion polypeptide. The immune response can include the generation of antibodies; activation of cytotoxic T lymphocytes (CTL) against cells presenting peptides derived from the polypeptides or other mechanisms well known in the art. See *e.g.* Paul *Fundamental Immunology Third Edition* published by Raven press New York (incorporated herein by reference) for a description of immune response. Useful carriers are well known in the art, and include, for example, thyroglobulin, albumins such as human serum albumin, tetanus toxoid, and polyamino acids such as poly(D-lysine:D-glutamic acid). The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, and further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art.

The compositions are suitable for single administrations or a series of administrations. When given as a series, inoculations subsequent to the initial administration are given to boost the immune response and are typically referred to as booster inoculations.

The vaccine compositions of the invention are intended for parenteral, topical, oral or local administration. Preferably, the pharmaceutical compositions are administered parenterally, *e.g.*, intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration that comprise a solution of the agents described above dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile

solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient and more preferably at a concentration of 25%-75%.

For aerosol administration, the polypeptides are preferably supplied in finely divided form along with a surfactant and propellant. The surfactant should be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. A carrier can also be included, as desired, as with, *e.g.*, lecithin for intranasal delivery.

The amount of vaccine administered to the patient will vary depending upon the composition being administered, the physiological state of the patient and the manner of administration.

Live attenuated recombinant viruses which include the deletion polypeptide, such as recombinant vaccinia or adenovirus vectors, are convenient alternatives as vaccines because they are inexpensive to produce and are easily transported and administered. Vaccinia vectors and methods useful in immunization protocols are described, for example, in U.S. Patent No. 4,722,848, incorporated herein by reference.

Deletion sequences and subsequences of this invention may also be used in methods of genetic immunization. Briefly, genetic immunization involves transfecting

cells *in vivo* with nucleic acids encoding pathogen specific antigens. The transformed host cells then express the antigen thereby stimulating the host immune system.

In the present invention, antigen-encoding deletion region sequences are used to transform mammalian host cells thereby resulting in the expression of the antigen by the host. This provokes an immune response by the host against the expressed antigen thereby conferring immunity on the host. Methods of genetic immunization are well known to those of skill in the art (see, *e.g.*, Wang *et al. Proc. Natl. Acad. Sci. USA*, 90: 4156-4160 (1993); Ulmer *et al.*, *Science*, 259: 1745-1749 (1993); Fynan *et al. DNA Cell Biol.*, 12: 785-789 (1993); Fynan *et al. Proc. Natl. Acad. Sci. USA*, 90: 11478-11482 (1993); Robinson *et al. Vaccine*, 11: 957-960 (1993); and Martinon *et al. Eur. J. Immunol.*, 23: 1719-1722 (1993), which are incorporated herein by reference.

VI. Use of Promoters within Deletion Sequences for Expression of Recombinant Proteins

Bacille Calmette-Guérin (BCG) contains all three deletions (BCGΔ1, BCGΔ2, and BCGΔ3) and yet is able to grow and reproduce indicating that the sequences contained within the deletion are not essential for bacterial viability. These deletion regions therefore make good target sites for the insertion of heterologous DNA as mycobacteria are tolerant of disruption of the native genome in these regions. The BCGΔ1, BCGΔ2, and BCGΔ3 deletion regions therefore provide suitable target sites for the incorporation of expression cassettes and the subsequent expression of exogenous gene products. The expression cassettes typically comprise a nucleic acid sequence under the control of a promoter. The promoter may be either constitutive or inducible. The cassette may additionally comprise a selectable marker such as an antibiotic resistance gene, a gene encoding a fluorescent marker (*e.g.* green fluorescent protein), or a gene encoding an enzymatic marker (*e.g.* ß-galactosidase).

Alternatively, genes under the control of endogenous promoters may be used as well. In one embodiment, reporter genes under the control of endogenous promoters found within the deletion sequences may be inserted at the deletion sites. These reporter genes may be utilized as an assay for antimycobacterial compounds that act by inhibiting transcription or translation of deletion sequences. Assaying for the

reporter gene product in the presence of an antimycobacterial compound provides a measure of efficacy of that compound in upregulating or downregulating deletion sequence genes. Methods of use of mycobacterial reporter gene assays to screen for drug activity are described by Cooksey *et al.*, *Antimicrob. Agents Chemother.*, 37: 1348-1352 (1993), and Jacobs *et al.*, *Science*, 260: 819-822 (1993) which are incorporated herein by reference.

EXAMPLES

The following examples are offered by way of illustration, not by way of limitation.

Example 1

Identification of Virulence-Attenuating Deletions

Bacterial Culture

All strains of Mycobacteria used in this study were maintained in 7H9 (Difco, Detroit Michigan, USA) media supplemented with OADC (BBL) or were grown on 7H11 agar supplemented with oleic acid albumin dextrose complex (OADC). *Escherichia coli* (strain DH5α or NM554) was used as a host for all recombinant plasmids and cosmids. *E. coli* was maintained in LB medium with or without agar. Carbenicillin (100 μg/ml) was used in place of ampicillin for the selection of all *E. coli* plasmids.

Extraction of High Molecular Weight DNA

High molecular weight chromosomal DNA was prepared by diluting a late log phase culture of the respective mycobacterium 1:10 into a liter of 7H9 medium containing 1.5% glycine and continuing growth for 4 to 5 days. The cells were then harvested by centrifugation, washed once in TE (pH 8.0) and resuspended in 4 ml of 25% sucrose in 10X TE. 100 μg of lysozyme was added and the preparation was incubated at 37°C for 2 hr followed by the addition of 100 μg of proteinase K and sarkosyl to a concentration of 1% weight/volume. Following overnight incubation at 65°C the mixture was extracted 4 times with chloroform isoamyl alcohol 24:1, once with phenol/chloroform (1:1), and twice again with chloroform isoamyl alcohol. The resulting high molecular weight DNA was then run on a CsCl gradient as described by

Hull *et al. Infect. Immun.*, 33: 933-938 (1981), which is incorporated herein by reference, and subsequently dialyzed against 4 changes of TE. BCG DNA was physically sheared by passage through a 22 gauge needle until an average size of 3-10 kb was obtained (20-25 passages). This DNA was then biotinylated using photobiotin (Clonetech, Palo Alto, California, USA) according to the method of Straus and Ausubel, *Proc. Natl. Acad. Sci. USA*, 87: 1889-1893 (1990), which is incorporated herein by reference.

DNA Subtraction

DNA subtraction was carried out between virulent *M. tuberculosis* H37Rv and avirulent BCG. H37R chromosomal DNA was selected because it was the most readily available chromosomal DNA from a virulent strain. In addition, *M. bovis* and *M tuberculosis* H37Rv are highly homologous.

M. bovis/M. tuberculosis specific probes were generated by the method of Straus and Ausubel, *supra.* with the following modifications. Sheared and biotinylated BCG DNA was used in a 10:1 excess for each round of subtraction. Wild type *M. tuberculosis* H37Rv DNA was digested with Sau3A to an average size of 1 kb. Hybridization conditions were 1M NaCl and 65 °C for 18 hours. Following five cycles (successive denaturation and reassociations) of subtraction, Sau3A1 adaptors (GACACTCTCGAGACATCACCGTCC and GATCGGACGGTGATGTCTCGAGAGTG were ligated to the subtraction product and amplified in a PCR reaction for 35 cycles (30 sec at 95°C, 30 sec at 55°C, and 3 min at 72°C). The *M. tuberculosis/M. bovis* specific probes were radiolabeled by using one strand of the adaptor (GACACTCTCGAGACATCACCGTCC) as a primer and labeling with ^{32}P dCTP using the Klenow fragment of DNA polymerase.

An *M. bovis* cosmid library was constructed in the BamH1 site of sCOS (Stratagene, La Jolla California, USA) with subsequent *in vitro* packaging and infection of *E. coli* strain NM554 (Stratagene). 600 colonies were picked to Nytran circular membranes and the membranes prepared according to the method of Grunstein and Hogness, *Proc. Natl. Acad. Sci. USA*, 72: 3961 (1975), which is incorporated herein by reference. These filters were then probed using the BCG subtracted probe and positive clones selected for further analysis. Cosmid DNA was prepared from selected clones by the method of Birnboim and Doly, *Nucleic Acids. Res.*, 7: 1513 (1973) which is

incorporated herein by reference. Restriction fragments that hybridize with the MTB/MBV specific probe were further subcloned into pGEM7z or pGEM5z (Promega, Madison, Wisconsin, USA) for deletion analysis.

Plasmid DNA for DNA sequencing was prepared using Qiagen minicolumns (Qiagen Inc. Chatsworth California, USA) and sequenced by the method of Henikoff, *Gene*, 28: 351-359 (1984), which is incorporated herein by reference, using the Erase A Base System (Promega). DNA sequencing reactions were run using a Perkin Elmer 9600 thermocycler and analyzed on an automated ABI sequencer. Analysis and assembly of contiguous DNA sequence was done using the ABI analysis software and SeQuencher sequence analysis software by Gene Clones Corp (Ann Arbor, Michigan, USA).

Deletion Region 1 (BCGΔ1)

Sequence analysis of over 16 kb of MBV region 1 and homologous regions in BCG revealed the precise junctions for the deletion in BCG. Eight open reading frames were identified with codon usage biases matching that of known MTB and MBV genes (see map Figure 4). The potential start and stop codons and predicted maximum protein coding capacity are listed in Figure 4. Consensus ribosomal binding site sequences were found near potential start codons for seven of eight open reading frames. TBLASTN and FASTA sequence homology analysis with each potential ORF-encoded protein revealed significant homologies for 3 of 8 open reading frames in region 1.

Most notable is the ORF1C homology to an unpublished and uncharacterized sequence listed in Genbank as *M. tuberculosis* antigen esat6. A 65 base pair repeated overlapping (repeated ~2 1/2 times) sequence was also recognized within the ORF1C (esat6) open reading frame. Also noteworthy are the significant homologies identified between ORF1H and bacterial serine proteases including *B. subtilus* subtilisin. Of the eight recognized open reading frames, four (ORFs 1B, 1C, 1D, and 1E) are located entirely within the 9 kb region deleted in BCG. One ORF traverses the BCG deletion junction in virulent *M. bovis*.

DNA probes from the 9 kb deletion in region 1 demonstrated that this region is absent in all BCG substrains and present in all virulent MBV and MTB strains tested. Furthermore, restriction fragment patterns observed in Southern blot analysis

with region 1 probes are non-polymorphic and identical in virulent MBV and MTB. This region has far fewer direct and indirect repeats than the regions 2 (BCGΔ2) and 3 (BCGΔ3) characterized below.

The sequence of a small region, estimated to be less than 20 bp between basepair coordinates 10654 and 10664 in region 1 has been recalcitrant to automated sequencing. Therefore, pending sequence confirmation, the base pair coordinates given in the region 1 map (Figure 4) are approximations. The precise sequence determination is likely to effect the Orf1E open reading frame.

Deletion Region 2 (BCGΔ2)

Sequence analysis of over 15 kb of MBV region 2 and homologous regions in BCG revealed the precise junctions for an 11 kb deletion in BCG. Thirteen open reading frames were identified with codon usage biases matching that of known MTB and MBV genes (see map Figure 5). The potential start and stop codons and predicted maximum protein coding capacity are also shown in Figure 5. Candidate consensus sequences resembling ribosomal binding sites were found near potential start codons for eight open reading frames. Of the thirteen open reading frames recognized in BCGΔ2, nine are located entirely within the 11 kb region deleted in most BCG strains while ORF2B2 and ORF2I traverse the deletion junctions.

TBLASTN and FASTA sequence homology analysis with each potential ORF-encoded protein revealed significant homologies for five open reading frames in BCGΔ2. A protein encoded by ORF2C exhibits striking similarity to the *E. coli* iciA protein which is thought to play a role in inhibiting and regulating the initiation of chromosomal replication. The iciA protein product is a member of the large LysR family of transcriptional regulatory proteins. Orf2F is highly homologous to an *S. typhimurium* ribonucleotide diphosphate reductase and a region of the *E. coli* and *S. typhimurium* proUVWX operon. Orf2H was found to have significant homology to *E. coli* and *S. typhimurium* permeases involved in aromatic amino acid transport and a eukaryotic cell retroviral receptor.

The Orf2G encoded protein was identical to the MTB mpt64 gene previously thought to encode a secreted antigen which is specifically expressed by MTB

and not BCG strains. Recent analysis of mpt64 expression revealed that three BCG substrains do express mpt64 (Moreau, Tokyo, Russian). Probes specific for mpt64 or other non-repetitive parts of region 2 hybridized to all MTB strains tested and the same three BCG substrains shown to express mpt64. Of interest is the finding that these three BCG substrains are derived from the original Pasteur strain prior to 1925. The current Pasteur strain and all strains derived from the original Pasteur strain after 1925, including the Connaught strain used in the subtractive analysis in this study, are deleted in the 11 kb DNA segment contained within BCGΔ2. These data indicate that an additional mutational event deleting the 11 kb segment of region 2, occurred in the BCG Pasteur strain sometime after 1925.

Southern blot analysis with probes from different segments of region 2 revealed a repetitive element located within a 2 kb segment (8-10 kb) of region 2. This repetitive element is ubiquitous in all tubercle bacilli tested. This element provides a marker suitable for RFLP analysis of mycobacterial strains.

Deletion Region 3 (BCGΔ3)

Sequence analysis of the almost 11 kb region 3 sequence and comparison to a homologous region in BCG precisely identified the deletion junctions for BCG. Twelve potential open reading frames were recognized in the region 3 sequence, seven of which are entirely located within the 9 kb region deleted in BCG. At least 9 ORFs in BCGΔ3 exhibit codon usage preferences comparable to that of the tubercle bacilli. Sequence homology analysis of presumptive protein sequences encoded by six open reading frames in region 3 revealed highly significant homology to listed sequences. Orfs3B, 3D, and 3E exhibit homology to phage sequences, suggesting a phage derivation for 4 or more kb of DNA in region 3. Homology to putative open reading frames in two *M. leprae* cosmids was also observed including homology to a putative *bid* gene encoding a protein involved in biotin synthesis. Also of interest was homology between ORF3A and an MTB sequence (*mce*) associated with cell invasion and intracellular survival.

Southern blot analysis with segments of region 3 deleted in BCG revealed that prototype lab strains of virulent MBV and MTB all carry deletion region 3 DNA. However, clinical isolates from PHRI are highly polymorphic or deleted in region 3.

This region contains many large direct and indirect repeats and, as mentioned above, at least 2 ORFs are homologous to phage sequences including homology to DNA invertases or recombinases. The repetitive nature of this region and the possible presence of a DNA recombinase could explain the polymorphisms observed in this region.

The sequence of a small region, estimated to be much less than 200 bp and located close to 9400 bp in Figure 3, was recalcitrant to automated sequencing and remains to be determined. Therefore, the base pair coordinates given in the region 3 map (Figure 6) 3' to the 9kb marker are approximations. The precise sequence determination of region is likely to effect the length of open reading frames 3H and 3L.

The foregoing subtractive analysis identified 3 regions in virulent *M. bovis* and *M. tuberculosis* prototype strains which are deleted in the avirulent BCG strain. The deletion located in region 2 may not have arisen in the original BCG Pasteur strain as this region is only deleted in strains derived from the original Pasteur strain after 1925. Region 3 is present in virulent MTB and MBV lab prototype strains (H37Rv, Erdman) and is highly polymorphic and at least partially deleted in the majority of MTB clinical isolates tested. Region 1 is apparently conserved and intact in all virulent MBV and MTB strains tested to date while all avirulent BCG strains tested to date are missing approximately 9kb from region 1.

Example 2

Screening and Identification of an Avirulent Mycobacterium

The ^{32} P labeled subtraction probe obtained in Example 1, was used to probe EcoRI and BamHI restricted chromosomal DNAs from BCG Connaught, *Mycobacterium bovis*, and various strains of *Mycobacterium tuberculosis* in a Southern blot. The hybridization was performed at 70°C in 6X SSC overnight.

The resulting Southern blot is illustrated in Figure 8. The probe showed no labeling of BCG reflecting the presence of all three deletions, while the other strains were labeled.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. A marker for an avirulent mycobacterium, said marker comprising a first nucleic acid that specifically hybridizes under stringent conditions with a second nucleic acid or a complement of said second nucleic acid where said second nucleic acid or complement of said second nucleic acid is selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3.

2. The marker of claim 1, wherein said marker specifically hybridizes under stringent conditions to a nucleic acid from BCG, but not to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or where said marker specifically hybridizes under stringent conditions to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, but not to a nucleic acid from BCG.

3. The marker of claim 2, wherein said marker comprises a subsequence of a nucleic acid where said nucleic acid is selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3.

4. The marker of claim 2, wherein said marker is selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3.

5. The marker of claim 2, wherein said marker comprises a nucleic acid having at least 90 percent sequence identity with a sequence selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3.

6. The marker of claim 2, wherein said marker comprises a radioactive nucleotide probe.

7. The marker of claim 2, wherein said subsequence is a sequence selected from an open reading frame of a deletion, said deletion being selected from the group consisting of BCGΔ1, BCGΔ2, BCGΔ3.

8. A polypeptide encoded by a subsequence of a deletion sequence selected from the group consisting of BCGΔ1, BCGΔ2, and BCGΔ3.

9. The polypeptide of claim 8, wherein the subsequence is selected from an open reading frame (ORF) of a deletion, said deletion being selected from the group consisting of BCGΔ1, BCGΔ2, BCGΔ3.

10. An antibody that binds specifically to the polypeptide of claim 8.

11. A recombinant cell comprising a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of said second nucleic acid where said second nucleic acid or complement of said second nucleic acid is selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3.

12. The recombinant cell of claim 11, wherein the cell is a Mycobacterium.

13. The cell of claim 11, wherein the cell expresses a polypeptide encoded by an intact open reading frame from BCGΔ1, BCGΔ2, and BCGΔ3.

14. The cell of claim 11, wherein said cell is a mycobacterium having one or more deletions in the genomic regions selected from the group consisting of BCGΔ1, BCGΔ2, and BCGΔ3, wherein said deletions result in the attenuation of an otherwise virulent strain of mycobacterium and wherein said deletions are present in up to two of said regions.

15. The mycobacterium of claim 14, wherein said deletions comprise a deletion selected from the group consisting of BCGΔ1, BCGΔ2, and BCGΔ3.

16. A method of distinguishing between an attenuated and a virulent mycobacterium, said method comprising detecting the presence or absence of a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of said second nucleic acid where said second nucleic acid or complement of said second nucleic acid is selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3.

17. The method of claim 16, wherein said first nucleic acid specifically hybridizes under stringent conditions to a nucleic acid from BCG, but not to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or where said first nucleic acid specifically hybridizes under stringent conditions to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, but not to a nucleic acid from BCG.

18. The method of claim 17, wherein said first sequence is amplified prior to detection.

19. The method of claim 17, wherein said first sequence is amplified by the polymerase chain reaction.

20. A method of claim 17, wherein said detecting comprises a Southern blot.

21. A method of claim 17, wherein said detecting comprises detecting a polypeptide encoded by said first nucleic acid.

22. The method of claim 21, wherein the polypeptide is encoded by an intact open reading frame of a nucleotide sequence selected from the group consisting of BCGΔ1, BCGΔ2, and BCGΔ3.

23. The method of claim 21, wherein the polypeptide is visualized by antibody hybridization.

24. A method for determining whether an attenuated or a virulent Mycobacterium is present in a sample comprising:

providing a first nucleic acid that hybridizes under stringent conditions with a second nucleic acid or a complement of said second nucleic acid where said second nucleic acid or complement of said second nucleic acid is selected from the group consisting of BCGΔ1a, BCGΔ1b, BCGΔ2a, BCGΔ2b, BCGΔ3a, BCGΔ3b, BCGΔ1ab, BCGΔ2ab, BCGΔ3ab, BCGΔ1, BCGΔ2, and BCGΔ3; and

hybridizing said first nucleic acid to the biological sample.

25. The method of claim 24, wherein said first nucleic acid specifically hybridizes under stringent conditions to a nucleic acid from BCG, but not to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or where said first nucleic acid specifically hybridizes under stringent conditions to a nucleic acid from *Mycobacterium tuberculosis* or *Mycobacterium bovis*, but not to a nucleic acid from BCG.

26. A method of producing an attenuated Mycobacterium species, said method comprising deleting from the genomic DNA of a virulent mycobacterium a first nucleic acid that specifically hybridizes under stringent conditions with a second nucleic acid or a complement of said second nucleic acid where said second nucleic acid or complement of said second nucleic acid is selected from the group consisting of BCGΔ1, BCGΔ2, and BCGΔ3.

FIGURE 1-1


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GAATTCCTGC GCACCCTGAT CCTGTCGCTG GTGGCAATGA CTCATCCAGA TCAGGTGAAT      60

CTCCTGCTCA CCGACTTCAA AGGTGGTTCA ACCTTCCTGG GAATGGAAAA GCTTCCGCAC     120

ACTGCCGCTG TCGTCACCAA CATGGCCGAG GAAGCCGAGC TCGTCAGCCG GATGGGCGAG     180

GTGTTGACCG GAGAACTCGA TCGGCGCCAG TCGATCCTCC GACAGGCCGG GATGAAAGTC     240

GGCGCGGCCG GAGCCCTGTC CGGCGTGGCC GAATACGAGA AGTACCGCGA ACGCGGTGCC     300

GACCTACCCC CGCTGCCAAC GCTTTTCGTC GTCGTCGACG AGTTCGCCGA GCTGTTGCAG     360

AGTCACCCGG ACTTCATCGG GCTGTTCGAC CGGATCTGCC GCGTCGGGCG GTCGCTGAGG     420

GTCCATCTGC TGCTGGCTAC CCAGTCGCTG CAGACCGGCG GTGTTCGCAT CGACAAACTG     480

GAGCCAAACC TGACATATCG AATCGCATTG CGCACCACCA GCTCTCATGA ATCCAAGGCG     540

GTAATCGGCA CACCGGAGGC GCAGTACATC ACCAACAAGG AGAGCGGTGT CGGGTTTCTC     600

CGGGTCGGCA TGGAAGACCC GGTCAAGTTC AGCACCTTCT ACATCAGTGG GCCATACATG     660

CCGCCGGCGG CAGGCGTCGA AACCAATGGT GAAGCCGGAG GGCCCGGTCA ACAGACCACT     720

AGACAAGCCG CGCGCATTCA CAGGTTCACC GCGGCACCGG TTCTCGAGGA GGCGCCGACA     780
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FIGURE 1-2


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CCGTGACCCG CGCCGGCGAC GATGCAAAGC GCAGCGATGA GGAGGAGCGG CGCCAACGGC    840
CCGCGCCGGC GACGATGCAA AGCGCAGCGA TGAGGAGGAG CGGCGCGCAT GACTGCTGAA    900
CCGGAAGTAC GGACGCTGCG CGAGGTTGTG CTGGACCAGC TCGGCACTGC TGAATCGCGT    960
GCGTACAAGA TGTGGCTGCC GCCGTTGACC AATCCGGTCC CGCTCAACGA GCTCATCGCC   1020
CGTGATCGGC GACAACCCCT GCGATTTGCC CTGGGGATCA TGGATGAACC GCGCCGCCAT   1080
CTACAGGATG TGTGGGGCGT AGACGTTTCC GGGGCCGGCG GCAACATCGG TATTGGGGGC   1140
GCACCTCAAA CCGGGAAGTC GACGCTACTG CAGACGATGG TGATGTCGGC CGCCGCCACA   1200
CACTCACCGC GCAACGTTCA GTTCTATTGC ATCGACCTAG GTGGCGGCGG GCTGATCTAT   1260
CTCGAAAACC TTCCACACGT CGGTGGGGTA GCCAATCGGT CCGAGCCCGA CAAGGTCAAC   1320
CGGGTGGTCG CAGAGATGCA AGCCGTCATG CGGCAACGGG AAACCACCTT CAAGGAACAC   1380
CGAGTGGGCT CGATCGGGAT GTACCGGCAG CTGCGTGACG ATCCAAGTCA ACCCGTTGCG   1440
TCCGATCCAT ACGGCGACGT CTTTCTGATC ATCGACGGAT GGCCCGGTTT TGTCGGCGAG   1500
TTCCCCGACC TTGAGGGGCA GGTTCAAGAT CTGGCCGCCC AGGGGCTGGG GTTCGGCGTC   1560
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FIGURE 1-3


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CACGTCATCA TCTCCACGCC ACGCTGGACA GAGCTGAAGT CGCGTGTTCG CGACTACCTC    1620

GGCACCAAGA TCGAGTTCCG GCTTGGTGAC GTCAATGAAA CCCAGATCGA CCGGATTACC    1680

CGCGAGATCC CGGCGAATCG TCCGGGTCGG GCAGTGTCGA TGGAAAAGCA CCATCTGATG    1740

ATCGGCGTGC CCAGGTTCGA CGGCGTGCAC AGCGCCGATA ACCTGGTGGA GGCGATCACC    1800

GCGGGGGTGA CGCAGATCGC TTCCCAGCAC ACCGAACAGG CACCTCCGGT GCGGGTCCTG    1860

CCGGAGCGTA TCCACCTGCA CGAACTCGAC CCGAACCCGC CGGGACCAGA GTCCGACTAC    1920

CGCACTCGCT GGGAGATTCC GATCGGCTTG CGCGAGACGG ACCTGACGCC GGCTCACTGC    1980

CACATGCACA CGAACCCGCA CCTACTGATC TTCGGTGCGG CCAAATCGGC CAAGACGACC    2040

ATTGCCCACG CGATCGCGCG CGCCATTTGT GCCCGAAACA GTCCCCAGCA GGTGCGGTTC    2100

ATGCTCGCGG ACTACCGCTC GGGCCTGCTG GACGCGGTGC CGGACACCCA TCTGCTGGGC    2160

GCCGGCGCGA TCAACCGCAA CAGCGCGTCG CTAGACGAGG CCGCTCAAGC ACTGGCGGTC    2220

AACCTGAAGA AGCGGTTGCC GCCGACCGAC CTGACGACGG CGCAGCTACG CTCGCGTTCG    2280

TGGTGGAGCG GATTTGACGT CGTGCTTCTG GTCGACGATT GGCACATGCA GCCGTGGGTG    2340
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FIGURE 1-4


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CCGCCGGGGG GATGCCGCCG ATGGCACCGC TGGCCCCGTT ATTGCCGGCG GCGGCAGATA    2400

TCGGGTTGCA CATCATTGTC ACCTGTCAGA TGAGCCAGGC TTACAAGGCA ACCATGGACA    2460

AGTTCGTCGG CGCCGCATTC GGGTCGGGCG CTCCGACAAT GTTCCTTTCG GGCGAGAAGC    2520

AGGAATTCCC ATCCAGTGAG TTCAAGGTCA AGCGGCGCCC CCCTGGCCAG GCATTTCTCG    2580

TCTCGCCAGA CGGCAAAGAG GTCATCCAGG CCCCCTACAT CGAGCCTCCA GAAGAAGTGT    2640

TCGCAGCACC CCCAAGCGCC GGTTAAGATT ATTTCATTGC CGGTGTAGCA GGACCCGAGC    2700

TCAGCCCGGT AATCGAGTTC GGGCAATGCT GACCATCGGG TTTGTTTCCG GCTATAACCG    2760

AACGGTTTGT GTACGGGATA CAAATACAGG GAGGGAAGAA GTAGGCAAAT GGAAAAAATG    2820

TCACATGATC CGATCGCTGC CGACATTGGC ACGCAAGTGA GCGACAACGC TCTGCACGGC    2880

GTGACGGCCG GCTCGACGGC GCTGACGTCG GTGACCGGGC TGGTTCCCGC GGGGGCCGAT    2940

GAGGTCTCCG CCCAAGCGGC GACGGCGTTC ACATCGGAGG GCATCCAATT GCTGGCTTCC    3000

AATGCATCGG CCCAAGACCA GCTCCACCGT GCGGGCGAAG CGGTCCAGGA CGTCGCCCGC    3060

ACCTATTCGC AAATCGACGA CGGCGCCGCC GGCGTCTTCG CCTAATAGGC CCCCAACACA    3120
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FIGURE 1-5

TCGGAGGGAG TGATCACCAT GCTGTGGCAC GCAATGCCAC CGGAGCTAAA TACCGCACGG 3180

CTGATGGCCG GCGCGGGTCC GGCTCCAATG CTTGCGGCGG CCGCGGGATG GCAGACGCTT 3240

TCGGCGGCTC TGGACGCTCA GGCCGTCGAG TTGACCGCGC GCCTGAACTC TCTGGGAGAA 3300

GCCTGGACTG GAGGTGGCAG CGACAAGGCG CTTGCGGCTG CAACGCCGAT GGTGGTCTGG 3360

CTACAAACCG CGTCAACACA GGCCAAGACC CGTGCGATGC AGGCGACGGC GCAAGCCGCG 3420

GCATACACCC AGGCCATGGC CACGACGCCG TCGCTGCCGG AGATCGCCGC CAACCACATC 3480

ACCCAGGCCG TCCTTACGGC CACCAACTTC TTCGGTATCA ACACGATCCC GATCGCGTTG 3540

ACCGAGATGG ATTATTTCAT CCGTATGTGG AACCAGGCAG CCCTGGCAAT GGAGGTCTAC 3600

CAGGCCGAGA CCGCGGTTAA CACGCTTTTC GAGAAGCTCG AGCCGATGGC GTCGATCCTT 3660

GATCCCGGCG CGAGCCAGAG CACGACGAAC CCGATCTTCG GAATGCCCTC CCCTGGCAGC 3720

TCAACACCGG TTGGCCAGTT GCCGCCGGCG GCTACCCAGA CCCTCGGCCA ACTGGGTGAG 3780

ATGAGCGGCC CGATGCAGCA GCTGACCCAG CCGCTGCAGC AGGTGACGTC GTTGTTCAGC 3840

CAGGTGGGCG GCACCGGCGG CGGCAACCCA GCCGACGAGG AAGCCGCGCA GATGGGCCTG 3900

FIGURE 1-6

CTCGGCACCA GTCCGCTGTC GAACCATCCG CTGGCTGGTG GATCAGGCCC CAGCGCGGGC 3960

GCGGGCCTGC TGCGCGCGGA GTCGCTACCT GGCGCAGGTG GGTCGTTGAC CCGCACGCCG 4020

CTGATGTCTC AGCTGATCGA AAAGCCGGTT TGCCCCCTCG GTGATGCCGG CGGCTGCTGC 4080

CGGATCGTCG GCGACGGGTG GCGCCGCTCC GGTGGGTGCG GGAGCGATGG GCCAGGGTGC 4140

GCAATCCGGC GGCTCCACCA GGCCGGGTCT GGTCGCGCCG GCACCGCTCG CGCAGGAGCG 4200

TGAAGAAGAC GACGAGGACG ACTGGGACGA AGAGGACGAC TGGTGAGCTC CCGTAATGAC 4260

AACAGACTTC CCGGCCACCC GGGCCGGAAG ACTTGCCAAC ATTTTGGCGA GGAAGGTAAA 4320

GAGAGAAAGT AGTCCAGCAT GGCAGAGATG AAGACCGATG CCGCTACCCT CGCGCAGGAG 4380

GCAGGTAATT TCGAGCGGAT CTCCGGCGAC CTGAAAACCC AGATCGACCA GGTGGAGTCG 4440

ACGGCAGGTT CGTTGCAGGG CCAGTGGCGC GGCGCGGCGG GGACGGCCGC CCAGGCCGCG 4500

GTGGTGCGCT TCCAAGAAGC AGCCAATAAG CAGAAGCAGG AACTCGACGA GATCTCGACG 4560

AATATTCGTC AGGCCGGCGT CCAATACTCG AGGGCCGACG AGGAGCAGCA GCAGGCGCTG 4620

TCCTCGCAAA TGGGCTTCTG ACCCGCTAAT ACGAAAAGAA ACGGAGCAAA AACATGACAG 4680

FIGURE 1-7

AGCAGCAGTG GAATTTCGCG GGTATCGAGG CCGCGGCAAG CGCAATCCAG GGAAATGTCA 4740

CGTCCATTCA TTCCCTCCTT GACGAGGGGA AGCAGTCCCT GACCAAGCTC GCAGCGGCCT 4800

GGGGCGGTAG CGGTTCGGAG GCGTACCAGG GTGTCCAGCA AAAATGGGAC GCCACGGCTA 4860

CCGAGCTGAA CAACGCGCTG CAGAACCTGG CGCGGACGAT CAGCGAAGCC GGTCAGGCAA 4920

TGGCTTCGAC CGAAGGCAAC GTCACTGGGA TGTTCGCATA GGGCAACGCC GAGTTCGCGT 4980

AGAATAGCGA AACACGGGAT CGGGCGAGTT CGACCTTCCG TCGGTCTCGC CCTTTCTCGT 5040

GTTTATACGT TTGAGCGCAC TCTGAGAGGT TGTCATGGCG GCCGACTACG ACAAGCTCTT 5100

CCGGCCGCAC GAAGGTATGG AAGCTCCGGA CGATATGGCA GCGCAGCCGT TCTTCGACCC 5160

CAGTGCTTCG TTTCCGCCGG CGCCCGCATC GGCAAACCTA CCGAAGCCCA ACGGCCAGAC 5220

TCCGCCCCCG ACGTCCGACG ACCTGTCGGA GCGGTTCGTG TCGGCCCCGC CGCCGCCACC 5280

CCCACCCCCA CCTCCGCCTC CGCCAACTCC GATGCCGATC GCCGCAGGAG AGCCGCCCTC 5340

GCCGGAACCG GCCGCATCTA AACCACCCAC ACCCCCCATG CCCATCGCCG GACCCGAACC 5400

GGCCCCACCC AAACCACCCA CACCCCCCAT GCCCATCGCC GGACCCGAAC CGGCCCCACC 5460

FIGURE 1-8

CAAACCACCC ACACCTCCGA TGCCCATCGC CGGACCTGCA CCCACCCCAA CCGAATCCCA 5520

GTTGGCGCCC CCCAGACCAC CGACACCACA AACGCCAACC GGAGCGCCGC AGCAACCGGA 5580

ATCACCGGCG CCCCACGTAC CCTCGCACGG GCCACATCAA CCCCGGCGCA CCGCACCAGC 5640

ACCGCCCTGG GCAAAGATGC CAATCGGCGA ACCCCCGCCC GCTCCGTCCA GACCGTCTGC 5700

GTCCCCGGCC GAACCACCGA CCCGGCCTGC CCCCCAACAC TCCCGACGTG CGCGCCGGGG 5760

TCACCGCTAT CGCACAGACA CCGAACGAAA CGTCGGGAAG GTAGCAACTG GTCCATCCAT 5820

CCAGGCGCGG CTGCGGGCAG AGGAAGCATC CGGCGCGCAG CTCGCCCCCG GAACGGAGCC 5880

CTCGCCAGCG CCGTTGGGCC AACCGAGATC GTATCTGGCT CCGCCCACCC GCCCCGCGCC 5940

GACAGAACCT CCCCCCAGCC CCTCGCCGCA GCGCAACTCC GGTCGGCGTG CCGAGCGACG 6000

CGTCCGACCC CGATTTAGCC GCCCAACATG CCGCGGCGCA ACCTGATTCA ATTACGGCCG 6060

CAACCCACTG GCGGTCGTCG CCGCAAGCGT GCAGCGCCGG GATGCTCGAC GCGACACAAG 6120

AAATCCTTAA GGCCGGCGGC CAAGGGGCCG AAGGTGAAGA AGGTGAAGCC CCAGAAACCG 6180

AAGGCCACGA AGCCGCCCAA AGTGGTGTCG CAGCGCGGCT GGCGACATTG GGTGCATGCG 6240

FIGURE 1-9


```
TTGACGCGAA TCAACCTGGG CCTGTCACCC GACGAGAAGT ACGAGCTGGA CCTGCACGCT   6300
CGAGTCCGCC GCAATCCCCG CGGGTCGTAT CAGATCGCCG TCGTCGGTCT CAAAGGTGGG   6360
GCTGGCAAAA CCACGCTGAC AGCAGCGTTG GGGTCGACGT TGGCTCAGGT GCGGGCCGAC   6420
CGGATCCTGG CTCTAGACGC GGATCCAGGC GCCGGAAACC TCGCCGATCG GGTAGGGCGA   6480
CAATCGGGCG CGACCATCGC TGATGTGCTT GCAGAAAAAG AGCTGTCGCA CTACAACGAC   6540
ATCCGCGCAC ACACTAGCGT CAATGCGGTC AATCTGGAAG TGCTGCCGGC ACCGGAATAC   6600
AGCTCGGCGC AGCGCGCGCT CAGCGACGCC GACTGGCATT TCATCGCCGA TCCTGCGTCG   6660
AGGTTTTACA ACCTCGTCTT GGCTGATTGT GGGGCCGGCT TCTTCGACCC GCTGACCCGC   6720
GGCGTGCTGT CCACGGTGTC CGGTGTCGTG GTCGTGGCAA GTGTCTCAAT CGACGGCGCA   6780
CAACAGGCGT CGGTCGCGTT GGACTGGTTG CGCAACAACG GTTACCAAGA TTTGGCGAGC   6840
CGCGCATGCG TGGTCATCAA TCACATCATG CCGGGAGAAC CCAATGTCGC AGTTAAAGAC   6900
CTGGTGCGGC ATTTCGAACA GCAAGTTCAA CCCGGCCGGG TCGTGGTCAT GCCGTGGGAC   6960
AGGCACATTG CGGCCGGAAC CGAGATTTCA CTCGACTTGC TCGACCCTAT CTACAAGCGC   7020
```

FIGURE 1-10

AAGGTCCTCG AATTGGCCGC AGCGCTATCC GACGATTTCG AGAGGGCTGG ACGTCGTTGA 7080

GCGCACCTGC TGTTGCTGCT GGTCCTACCG CCGCGGGGGC AACCGCTGCG CGGCCTGCCA 7140

CCACCCGGGT GACGATCCTG ACCGGCAGAC GGATGACCGA TTTGGTACTG CCAGCGGCGG 7200

TGCCGATGGA AACTTATATT GACGACACCG TCGCGGTGCT TTCCGAGGTG TTGGAAGACA 7260

CGCCGGCTGA TGTACTCGGC GGCTTCGACT TTACCGCGCA AGGCGTGTGG GCGTTCGCTC 7320

GTCCCGGATC GCCGCCGCTG AAGCTCGACC AGTCACTCGA TGACGCCGGG GTGGTCGACG 7380

GGTCACTGCT GACTCTGGTG TCAGTCAGTC GCACCGAGCG CTACCGACCG TTGGTCGAGG 7440

ATGTCATCGA CGCGATCGCC GTGCTTGACG AGTCACCTGA GTTCGACCGC ACGGCATTGA 7500

ATCGCTTTGT GGGGGCGGCG ATCCCGCTTT TGACCGCGCC CGTCATCGGG ATGGCGATGC 7560

GGGCGTGGTG GGAAACTGGG CGTAGCTTGT GGTGGCCGTT GGCGATTGGC ATCCTGGGGA 7620

TCGCTGTGCT GGTAGGCAGC TTCGTCGCGA ACAGGTTCTA CCAGAGCGGC CACCTGGCCG 7680

AGTGCCTACT GGTCACGACG TATCTGCTGA TCGCAACCGC CGCAGCGCTG GCCGTGCCGT 7740

TGCCGCGCGG GGTCAACTCG TTGGGGGCGC CACAAGTTGC CGGCGCCGCT ACGGCCGTGC 7800

FIGURE 1-11

TGTTTTTGAC CTTGATGACG CGGGGCGGCC CTCGGAAGCG TCATGAGTTG GCGTCGTTTG 7860

CCGTGATCAC CGCTATCGCG GTCATCGCGG CCGCCGCTGC CTTCGGCTAT GGATACCAGG 7920

ACTGGGTCCC CGCGGGGGGG ATCGCATTCG GGCTGTTCAT TGTGACGAAT GCGGCCAAGC 7980

TGACCGTCGC GGTCGCGCGG ATCGCGCTGC CGCCGATTCC GGTACCCGGC GAAACCGTGG 8040

ACAACGAGGA GTTGCTCGAT CCCGTCGCGA CCCCGGAGGC TACCAGCGAA GAAACCCCGA 8100

CCTGGCAGGC CATCATCGCG TCGGTGCCCG CGTCCGCGGT CCGGCTCACC GAGCGCAGCA 8160

AACTGGCCAA GCAACTTCTC ATCGGATACG TCACGTCGGG CACCCTGATT CTGGCTGCCG 8220

GTGCCATCGC GGTCGTGGTG CGCGGGCACT TCTTTGTACA CAGCCTGGTG GTCGCGGGTT 8280

TGATCACGAC CGTCTGCGGA TTTCGCTCGC GGCTTTACGC CGAGCGCTGG TGTGCGTGGG 8340

CGTTGCTGGC GGCGACGGTC GCGATTCCGA CGGGTCTGAC GGCCAAACTC ATCATCTGGT 8400

ACCCGCACTA TGCCTGGCTG TTGTTGAGCG TCTACCTCAC GGTAGCCCTG GTTGCGCTCG 8460

TGGTGGTCGG GTCGATGGCT CACGTCCGGC GCGTTTCACC GGTCGTAAAA CGAACTCTGG 8520

AATTGATCGA CGGCGCCATG ATCGCTGCCA TCATTCCCAT GCTGCTGTGG ATCACCGGGG 8580

FIGURE 1-12

TGTACGACAC GGTCCGCAAT ATCCGGTTCT GAGCCGGATC GGCTGATTGG CGGTTCCTGA 8640

CAGAACATCG AGGACACGGC GCAGGTTTGC ATACCTTCGG CGCCCGACAA ATTGCTGCGA 8700

TTGAGCGTGT GGCGCGTCCG GTAAAATTTG CTCGATGGGG AACACGTATA GGAGATCCGG 8760

CAATGGCTGA ACCGTTGGCC GTCGATCCCA CCGGCTTGAG CGCAGCGGCC GCGAAATTGG 8820

CCGGCCTCGT TTTTCCGCAG CCTCCGGCGC CGATCGCGGT CAGCGGAACG GATTCGGTGG 8880

TAGCAGCAAT CAACAAGACC ATGCCAAGCA TCGAATCGCT GGTCAGTGAC GGGCTGCCCG 8940

GCGTGAAAGC CGCCCTGACT CGAACAGCAT CCAACATGAA CGCGGCGGCG GACGTCTATG 9000

CGAAGACCGA TCAGTCACTG GGAACCAGTT TGAGCCAGTA TGCATTCGGC TCGTCGGGCG 9060

AAGGCCTGGC TGGCGTCGCC TCGGTCGGTG GTCAGCCAAG TCAGGCTACC CAGCTGCTGA 9120

GCACACCCGT GTCACAGGTC ACGACCCAGC TCGGCGAGAC GGCCGCTGAG CTGGCACCCC 9180

GTGTTGTTGC GACGGTGCCG CAACTCGTTC AGCTGGCTCC GCACGCCGTT CAGATGTCGC 9240

AAAACGCATC CCCCATCGCT CAGACGATCA GTCAAACCGC CCAACAGGCC GCCCAGAGCG 9300

CGCAGGGCGG CAGCGGCCCA ATGCCCGCAC AGCTTGCCAG CGCTGAAAAA CCGGCCACCG 9360

PCT/US96/01938

FIGURE 1-13


```
AGCAAGCGGA GCCGGTCCAC GAAGTGACAA ACGACGATCA GGGCGACCAG GGCGACGTGC    9420
AGCCGGCCGA GGTCGTTGCC GCGGCACGTG ACGAAGGCGC CGGCGCATCA CCGGGCCAGC    9480
AGCCCGGCGG AGGCGTTCCC GCGCAAGCCA TGGATACCGG AGCCGGTGCC CGCCCAGCGG    9540
CGAGTCCGCT GGCGGCCCCC GTCGATCCGT CGACTCCGGC ACCCTCAACA ACCACAACGT    9600
TGTAGACCGG GCCTGCCAGC GGCTCCGTCT CGCACGCAGC GCCTGTTGCT GTCCTGGCCT    9660
CGTCAGGATG CGGCGGCCAG GGCCCGGTCG AGCAACCCGG TGACGTATTG CCAGTACAGC    9720
CAGTCCGCGA CGGCCACACG CTGGACGGCC GCGTCAGTCG CAGTGTGCGC TTGGTGCAGG    9780
GCAATCTCCT GTGAGTGGGC AGCGTAGGCC CGGAACGCCC GCAGATGAGC GGCCTCGCGG    9840
CCGGTAGCGG TGCTGGTCAT GGGCTTCATC AGCTCGAACC ACAGCATGTG CCGCTCATCG    9900
CCCGGTGGAT TGACATCCAC CGGCGCCGGC GGCAACAAGT CGAGCAAACG CTGATCGGTA    9960
GTGTCGGCCA GCTGAGCCGC CGCCGAGGGG TCGACGACCT CCAGCCGCGA CCGGCCCGTC   10020
ATTTTGCCGC TCTCCGGAAT GTCATCTGGC TCCAGCACAA TCTTGGCCAC ACCGGGATCC   10080
GAACTGGCCA ACTGCTCCGC GGTACCGATC ACCGCCCGCA GCGTCATGTC GTGGAAAGCC   10140
```

FIGURE 1-14

GCCCAGGCTT GCACGGCCAA AACCGGGTAG GTGGCACAGC GTGCAATTTC GTCAACCGGG 10200

ATTGCGTGAT CCGCGCTGGC CAAGTACACC TTATTCGGCA ATTCCATCCC GTCGGGTATG 10260

TAGGCCAGCC CATAGCTGTT GGCCACGACG ATGGAACCGT CGGTGGTCAC CGCGGTGATC 10320

CAGAAGAACC CGTAGTCGCC CGCGTTGTTG TCGGACGCGT TGAGCGCCGC CGCGATGCGT 10380

CGCGCCAACC GCAGCGCATC ACCGCGGCCA CGCTGGCGGG CGCTGGCAGC TGCAGTGGCG 10440

GCGTCGCGTG CCGCCCGAGC CGCCGACACC GGGATCATCG ACACCGGCGT ACCGTCATCT 10500

GCAGACTCGC TGCGATCGGG TTTGTCGATG TGATCGGTCG ACGGAGGGCG GGCAGGAGGT 10560

GCCGTCCGCG CCGAGGCCGC CCGCGTGCTC GGTGCCGCCG CCTTGTCCGA GGTAGCCACC 10620

TGCGTCCGCC CAGTGGCAGT ATGCGGACCC CGGAAAAAAA AAACTCGAGT GCGTTCTTCG 10680

GAGGTTTCCA ATTCTTGGAT TCCAGCACCC GCTCAGCGGT CTCGGCGACC AGACTGACAT 10740

TGGCCCCATG CGTCGCCGTG ACCAATGAAT TGATGGCGGT ATGGCGCTCA TCAGCATCCA 10800

GGCTAGAGTC ATTCTCCAGG ATATCGATCT CCCGTTGAGC GCCATCCACA TTATTGCCGA 10860

TATCGGATTT AGCTTGCTCA ATCAACCCGG CAATATGCCT GTGCCAGGTA ATCACCGTGG 10920

FIGURE 1-15

CGAGATAATC CTGCAGCGTC ATCAATTGAT TGATGTTTGC ACCCAGGGCG CCGTTGGCAG 10980

CATTGGCGGC GCCGCCGGAC CATAGGCCGC CTTCGAAGAC GTGGCCTTTC TGCTGGCGGC 11040

AGGTGTCCAA TACATCGGTG ACCCTTTGCA AAACCTGGCT ATATTCCTGG GCCCGGTCAT 11100

AGAAAGTGTC TTCATCGGCT TCCACCCAGC CGCCCGGATC CAGCATCTGT CTGGCATAGC 11160

TGCCCGTCGG CCTGGTAATA CTCATCCCCT ACTGCCCTCC CCAAACCGCC AGATCGCCTC 11220

GCGGATCACC GTCCGGTTGG CCTCCGGCAT TTCACGCCGG CTCGGCCGCT GGATCCACCC 11280

CGCGCCGGTA TTCGCAGTAA CCCGTTGAAT CCGCGCGCAT GATGCACCGC TTGGGCGATC 11340

AGCCGGGTGG TCACCTCGCT TGCGCTGGCC GCGCTGTCGC ACGGGGCGCT CGGTGGTAAC 11400

GGACGTCATA ATTAACCAGC GTAACCGAAC CTAAGACCAG CTAGCTGCGG CAATATTGGC 11460

GACCAGGACT ATGGCGCCCT CCGAACCCGG CCGATCCATG TCAAAACATT GACAATGCGT 11520

ACTCACGCCG TGTCGGGCGC GCTGAATGAC CGCATTGCGG CGCTCATTCG GTGCGTAGTC 11580

GCTACCACCG CAACAATGGG CTTAGGCCAT TCCTTCGTTC ATCGCGCGGG ACATGGCCGA 11640

TAACGCAGCG GTCAGCTGCT CGCCCGCCGC GTCGTTATAC GCGGACGCCG CGGCCTGCGC 11700

FIGURE 1-16

ATTGTGCAGC GCCTCGTTGA CCCGCTGAGC CGCCGCCTCG GCACCCAGCT TCTTCAGCAA 11760

ACCATCTTCG ATGCGCAGGC CGGTGAGCCA CTGGTGCCCA TTGATCGTCA CTTCGACGGT 11820

CTCGGCTTCG TCGGTGGCGC GGAAGGATCC GTTGTTCATC TGATTGAGCG TCCCGTCTAG 11880

GGCCGACTGA AACCGCGCCG CCAGCGTCAA CGCCCGGGCG ACATGCGGGT CCAATTCGTC 11940

CATGCTCACT TCGACTCCTT ACTGTCCTGG CGCCGACGGT TACCAATGAC GGCCTCGGTC 12000

CATGCCCGAT CCTCGGTGTA GAGCGCCTCG TCTTCCTGCT GAGAACCCTT GGACTTGGCG 12060

CCCCCTTGTC CCTGATGCGC GGCACCCATC GGCATTCCCA TGCCACCGCC GCCCAGCGCG 12120

GCGCCGCCGC CGGCCCTTCC CTGGCCTAAG CCGGCAATGT CACCAGCGCC AGCGGGCCGC 12180

ACCGATTCGG CGCCCCCGAT CGCGGATCCC AACGGCGCCG ACGGCACCCC GCCGCCTCCA 12240

CCGCCACCGA GCGATGCCGC TTTGACCGCC ACGTCGCCCG ACAGCGCTGC GGCTTCCCGC 12300

CCAGCCGACG TCAGCTGCGC CGCCGTGTCA GCCGGGAGGC CACCACCCGG CGATCCGGTA 12360

GGCGGAACCA TCGGTGCGGC TGGCATCCCG GTACCGGGAG TCACACCGGA GCCGTCAGAC 12420

GGCGGCATCA GGAAGCCAGG GATCAATCCC TGCTCTTGCG GAGGCGGGGC GGGTCGATCT 12480

FIGURE 1-17


```
TGATGGCGGG GGGGAGGCTT CGGCGGGTTT ACCGGTTCCA GGGCTGCCTT GTTGTTGTAT   12540

TCGGTCAGCA CCTTCTCCGA CCTCTGCTGA TACTCCGCGT ACACCGGGAG AATTTGGTCG   12600

CGGGCCGAAG GGTTTTCCGC GTAAAGCCGT TCGAGCCCGA CTATGTCTTC ATAAGTCGGA   12660

TGTTCCCGCC TAGCCCACAC GTGCAGCTGC GCGACATATT GAGCCTGCTT GGCCATCGCA   12720

GCGCTCAATT TGGCCATGTG GAGTATCCAT TGCCGGTGTT GATCGAGCGA AGCCTCGCAA   12780

GCGGTAGCCG CATCGCCTTC CCAGTTGTCA AACCCCCGGA ACCGCTTGAC GTCGCCTTGC   12840

AGCGTCAGGT TGAAAGTGTT CCACCCATCC GCAAAGTGCG CGAGCGATGC GCCTTGGTCG   12900

CCCGTTTCGA GCTTCCTTGC CGCTTCTTTG AGATCCATGA AGTTGGGTTC ACCGGCCGTG   12960

GCCACCCTCG GCGTATCGGT TAGTTCGGCC GAACTGTCCC CTCCGACGGC CCCGGCCGAT   13020

TCTGCCTGCA CAGTTCCTTC GCCGTCGTTG TCCAGCGCGG TCGCAGCCTC CTCATCAACC   13080

TCGCCATACG CCTTGGCCGC GTTGCGCAGC GAGGTCGCCA GACGCTGCCG CTCTTTGGCA   13140

CCGGCCGCCA GGTATTCCCG CATGTTGTCG GCGGACAATA CCAGCTGTTG GGCGGCGTTT   13200

TTAGCCGCCG TGAGTTCGCA CGGTGTGATG GGGACATCAG TCGGTGGGTC CGCCATCGGG   13260
```

FIGURE 1-18

GCCTCCACCT CGTTGGCCCT GTTCAAAATC TCTTGCTGAT CCACCGTCAC GGTCTGCGAC 13320

TGCGTCATAT CGGATCATCC TCCTTAGTGC TATAGCCATT ATCGTCGCTA AACTGAAAGG 13380

TTCCTGCACT AATTTGATGC CGCCCGTTCA TGCCGGCATC GCGAACGGAT CGCCCTACTT 13440

CGGCAGCGCC ATCTGGTAGC GGCTTTCCTC GGGTGGGGAA ACCCGGCGAA TCGGCAGCTG 13500

CCGATGCCGC GGGGTACCGA TCACATTGTG CCGCAGAATC ACCCGGTCAA TACCGGGATG 13560

CGGGCCGAGA TAGGTCGTCG CATTCGGCCA CGCCACCTTT ACCTCCTGCC CGATGTGTGC 13620

GCCGATCAAC CGGGCAAATT CCTCGAACTG TGGCCCGACT GTGACCATCG CACCTGCCGC 13680

CGCCGCACGC ACCACGAACT GGGTGAATGT CTGAGCGTCA CCCAGGTTGA GGGCGATGTC 13740

GACATCGTCG AAGGGCATGT AGACCGGGCA TCGGTTCACC GTCTCGCCGA CCAGTACCCC 13800

AGCTGACCCG ATCGGCAGCT GGCAGTGGCG GTTGGCCACC AGATGCTGGC CTTGCAGCGC 13860

GGGCCGCTGC CCGCCAAATA GGCGGGCGAA GCCCCTGGGT GTCTTGGGCT TGTCCGCCGT 13920

GGTCAGCAAC ACCGTGGACT GCGGGGCCAT CCCCGGCGCG ACCCGGACTC TGGTGATGGT 13980

GTGGTCCGCG CGCGCCGACC ACCATACATC CGGACCTCCG GGCGCCGCGT AGGCGGCAGT 14040

FIGURE 1-19

GTAGGCATCG CGCCCCTTGA TCATCGACCA TTTCTCCCGC ACAAAGCCGA TGTCGGTGGC 14100

GTGGTCGTAG TCATCGAAGC TGCGGCCACA CACCGCGTCG ACACCATGGC TAGCCAGTCG 14160

ATCGGCAATG CGCGTCGCGG ACGCCACCAA ATACCGGGCC AGTCCTGCGA CGCCTTCATC 14220

GCGGCGCTGC GCCGATTTGC GGGTGCGTTC CGGGTCGGCG CGCAGCACGA TCCAGGTCCG 14280

GCGGTTCGCC GGCGCCGGGT CTGTCCCGAT CACCTGCTGA TACAGACTCA CCACGTCCGG 14340

CGCTGCGGTA TTGCCGACGC GGTAGCCGGC TGAGACGATA TCGGCCTCCA AGTCGGGACA 14400

GTGCACCGAC AGGAGCTCCT CCACCAGTCC GGTGTCCAGC ATGTCGTCGG TGTGGGCTTG 14460

CCCGTCGACG ATGACCGTCG GCGTGAATGG TCGGGGAATG AGCTCGATTA CGGCGACCAG 14520

AAACTCGCCT TGCCAGCGCA CCGCAACGTG ATCTCCTGGC TTCACGGTGG CCCCGACCAC 14580

AGGTTCTGAC GAGGAATCCG GGGGCCGTCG GCGCCGCCGC AACCACGCGT ACACCGCCGC 14640

CACCCAGCCG GTGATCCGGC GGCCGTAGAA AGTGACCGTG GCCACGATGA CGCCCAACGA 14700

GGCCAGCGCA ATCCCCGCCC ACCAGTAGCG CGTCTCCAAG AATGCGATGA TGCATGGCGG 14760

GGCCAACGCG GAGGCAAGCA AGGCGTGCCC GGTGCTGAAC CGCAGCCCTA AAGGATTTCT 14820

FIGURE 1-20

CATCGGCGGC TCAGCGCCCG TCTAGCCAGC GCGCCCAGGC CCAGGGCCAA CGTAAGGCCG 14880

ACGGCCACCA ACGCCACAGC CGTAATCGGG CGACGATCGG GACCCGGCTC CACCACCGGG 14940

GGTGGAAGTC GTCTGACGTT GTATGGCGCC GAAGCAGGGC CGGGCGGAAT GTCCCACGTC 15000

AGCGCGGCCA CCGCATCGAT GACGCCGGCG CCGACCAGGT CGTCGACCCC GCCCCCGGGG 15060

TGTCTCGCGG TGGCGGTGAT CCGGTGGATG ATCTGCGCCG GCGTCAGGTC GGGGAACCGC 15120

TGCCGAAGCA GGGCCGCCAG ACCCGACACA TATGCCGCGG CAAACGAGGT GCCGGCGATG 15180

GGTACCGGCC CCTCCCGGCC TTGCAACGCA TTCACCGGTT CACCGGTGTC GCCGAGCGCG 15240

ACGATGTTTT CTGCGGGCGC GGCCACGTCC ACCCACGGTC CGTGCATCGA GAACGAGCTG 15300

GGCATCCCGG TCTGGCCGAT ACCGCCGACG CTTAACACCA GCGGTGCGTA CCACGCCGGG 15360

GTGACAACGG TCTGCACATT GTTCCAGCCG CGTGGGTCGC CGGGTGTGGA CGGGTCCGGC 15420

GCCGGATTCT GTACGCAATC GCCACCGGTG TTGCCGGCCG CGACCACCAC CACCACGCCT 15480

TTGACGTTGA CCGCATAGTC GATGGATGCA CCCAGTGAGG TTTCATCGAT CGGCCTGCTC 15540

ACCTTGTAGC AGGCGGCTTC ACTGATGTTG ATCACACCCA CGCCGAGGTT GGCGGCGTGC 15600

FIGURE 1-21

ACCACGGCGC GGGCAAGACT GCGGATGGAA CCGGCGGCCG GGGTGGCGTT GGGGTCATTC 15660

GGGTTGGCTT GTGAGCCGAC CGGTTCGAAG GCCTCAGACG TCTGACGTAG CGAGAGCAGT 15720

CGAGCGTCGG GCGCGACGCC GACGAACCCG TCGGTGGGCG CGGGCCGGCC CGCGATGATG 15780

GATGCTGTGA GAGTCCCATG GGCATCACAG TCAGACAGGC CGTTACCGGC CTGGTCGACG 15840

AAATCGCCGC CAGGTTCCGC CGGGACCCGT GGCGAAGCGT CGACACCGGT GTCGATCACC 15900

GCCACCGTCA CCCCGGCCCC GGTCGCGAAC TTGTGGGCAT CGGCCACGCC CAGATACGTG 15960

TTGCTCCACG GCGGATCGTG GAACCCGGAC CCCGGCAGCG TGGTGGGCGA CGCGCACAAA 16020

ACGCGCTGTT CGGTAGGCTG ATCCGGGCCC GCCACGTCGG GCGGCAACGC GCCCGGATCG 16080

ATCGGCGGTG GCGTGATGGC CGATGCGGGC GACGCGGTGA GCAACGCCAG CGCCACCGTG 16140

ATCAGAAAGA TACGGTGCAC TCCCAGAACA CTCCATTCGT TGAGATTCAT TGCGATTCAT 16200

TGAGCTGCGT TGCTACCTTG GGCCACTTGA CGGACCTGTG TGCATTTTAG ACGTAACGGC 16260

TGGGCAAACA ACGCTGTCAC GCCTGGGCTG GTCCGCCGCG CCGACCAGGG CGCGTAGGCG 16320

CTGTACCTGG ACCACGCCGG GACTCAACGG TTTTGCTACC GCACTAGCCG ATATGCGGCT 16380

FIGURE 1-22


```
GCTACCAAAC GATCGCGGCC ATGTCTCGGT TGTCTGAGCA CACGCTGCGT ATCGCGGCAT   16440
CGATGTCGGT GGCGGTGATG ATCTGCAGAT CCTGAACCGA TACCGGTTGG CCCGCACGTT   16500
TTTGCGCAAC CACCCGGGTG TCCCGGAACC CTTCGGCGCG TTCGATCACG TTGCGGGCGA   16560
ACCGACCGTT TTGCATAGCG TCGATACCGT GCTGCCCACT AGGGGTGGTG TAGTTACGGA   16620
TGGTGGTGAC CGCGTCGAGG AATACCTCCC GTGCGGCGTC ATCGAGCTGG CTGGCGCGCG   16680
GTGTAGCGTA GCGGTGTCCA ATCTCGACGA TCTCCACCGG CGAATAAGAC TCGAACCGCA   16740
GCTTTCGGTT GAACCGGCCA GCCAAACCCG GGTTCACGGT GAGGAATTCG GTACCCCGGG   16800
TTCGAAATCG ATAACTTGGA TCCGGAGAGC TCCCAACGCG TTGGATGCAT AGCTTGAGTA   16860
TTCTATAGTG TCACCTAAAT ACTTG                                         16885
```

FIGURE 2-1


```
GGATCCTCGG ACTGGCCGCG GTCGTGCTTG TGCACGAGTT CACCGAGGTC ATCGTCATCG      60
CCAACGGCGT GCGGGCCGGA CGCATCAAAC CACTTGCCGG GCCACCCAAG ACACCTGATC     120
GGACTATCCC GGGGTAGCGA CGCGCGGAAT CGTGGAGTGT GTTTGGACCA GCAATAGCGT     180
CACTGTGACG AAACAGCCGC CGTCTTCTGG AAGTTATACC CGGTTATACT ATCTGTATGA     240
AGACAGCTAT TTCTCTGCCG GATGAGACGT TCGATCGGGT ATCGCGGCGT GCGAGTGAGC     300
TCGGCATGAG TCGGTCCGAG TTCTTCACGA AGGCTGCGCA GCGCTACCTG CACGAGCTGG     360
ACGCCCAATT GCTCACGGGC CAGATCGACA GGGCTCTAGA GAGCATCCAT GGCACCGACG     420
AAGCGGAGGC CCTCGCCGTG GCCAACGCAT ACCGCGTGCT AGAAACCATG GACGATGAGT     480
GGTGATTAGT CGTGCCGAGA TCTACTGGGC TGACCTCGGG CCGCCATCAG GCAGTCAGCC     540
GGCGAAGCGC CGCCCGGTGC TCGTAATCCA GTCAGATCCG TACAACGCAA GTCGCCTTGC     600
CACTGTGATC GCAGCGGTGA TCACGTCCAA TACGGCGCTG GCGGCAATGC CCGGCAACGT     660
GTTCTTGCCC GCGACCACAA CGCGACTGCC ACGTGACTCG GTCGTCAACG TCACGGCGAT     720
TGTCACGCTC AACAAGACTG ACCTCACCGA CCGAGTTGGG GAGGTGCCAG CGAGCTTGAT     780
```

FIGURE 2-2

GCACGAGGTT GACCGAGGAC TTCGTCGCGT ACTGGACCTT TGACACTGCG CCACGCGACA 840

ATTCGTCACG GTGACGTTCC TGCTTGGTGT AAGCCCCCCC GCCGGGGGAA CTACTCGCCG 900

GAGGTGGTGT TGTGGGCAGG CTTGAGGGCA AGGTTGCATT CATTACGGGC GTGGCTCGGG 960

GTCAAGGCCG TTCGCATGCG GTCCGCCTAG CCGACGGCCA AGCGCGTGCG CTCGGCAAGG 1020

TCGATGTTGA GGCGTGCGGT GCGCTCGTTG GTGAGGTAGA AGTGTGGGGC CGTGACGTGC 1080

GTGACGATCG ACGGGTGTTT GTCGAGAGTC CTGCCGACGA GTTCGGCGCG TGCCGCCGCG 1140

TCGCGCGTCA GGGCATCCGT GTCGTAGGGC TGCCCGTTTC ACAGAGGGAA CTTGTCGAGC 1200

CCGAAGCCGG GTGCGCGGCG AGGCGCTCGG CTGCTGGCTC CCAGTAGACA TCTAGGCCTG 1260

CGTCGACTGC GGCTGCGGCA GCGTCGTGCT GGTGACGAGT GGCGTTGGTG TCCAGCGTGA 1320

TCGCAGTGGT GCCGGCGTGG TCGCGGGACA GGAAGTCCTC GACCGGTTTG TGATCACCCG 1380

GCCCGAGCCG AAACTGAATG CCCATCGTCG TGAAGTTCCT CTCGCATCGA CGCCTCGGTT 1440

CGTGTCATAA TACATGACAA ATCAATAGAC AAAAGGAAGA CAGGCTGCCC ATGGGAGTAA 1500

ATGTGCTCGC CTCGACCGTG TCGGGTGCGA TCGAGCGCTT GGGATTGACC TACGAGGAAG 1560

FIGURE 2-3


```
TCGGTGACAT CGTCGATGCC TCGCCGCGTT CCGTGGCGCG ATGGACCGCA GGTCAGGTGG    1620

TTCCCCAACG CCTCAACAAG CAACGACTTA TCGAGCTGGC CTATGTCGCC GACGCCCTCG    1680

CGGAAGTGCT GCCGCGTGAC CAGGCGAACG TGTGGATGTT TTCGCCGAAT CGGTTACTGG    1740

AACACCGCAA GCCTGCCGAC CTCGTGCGAG ACGGCGAGTA CCAACGCGTG TTGGCGCTCA    1800

TCGACGCGAT GGCGGAGGGA GTGTTCGTGT GAGCGATGCC CTCGATGAAG GGCTCGTCCA    1860

GCGTATCGAC GCACGCGGAA CAATTGAGTG GTCGGAAACG TGCTACCGGT ATACCGGCGC    1920

GCACCGTGAC GCCTTGTCCG GTGAGGGCGC GCGCAGATTC GGAGGCAGGT GGAATCCGCC    1980

GCTGCTCTTT CCGGCGATCT ATCTTGCTGA TTCCGCCCAA GCCTGCATGG TTGAGGTGGA    2040

ACGGGCGGCG CAAGCGGCTT CAACGACCGC AGAGAAGATG CTCGAGGCGG CCTACCGACT    2100

ACACACGATC GACGTCACGG ACCTGGCCGT CCTCGATCTG ACAACCCCGC AAGCTCGGGA    2160

AGCCGTGGGG CTCGAGAACG ACGACATCTA TGGCGACGAC TGGTCAGGGT GCCAGGCGGT    2220

CGGACATGCG GCCTGGTTCT TGCACATGCA AGGTGTCCTC GTGCCGGCGG CGGGCGGTGT    2280

CGGCCTCGTT GTCACCGCGT ATGAACAGCG AACTCGGCCG GGCCAACTAC AACTGCGACA    2340
```

FIGURE 2-4

AAGCGTCGAT CTGACGCCTG CTCTTTACCA AGAACTTCGA GCCACGTAGC TGGCCAGCTT 2400

GGCGCAGAGA AGGATGCCGC TGTGCCATGG TCATCGTAAG GAGCAACTCG CATCACTTAT 2460

AAGCCGATAA GCGACATTAT GTCAAGTGAA GCTGGTCGTA TTGGGTTAGC TGCGCCGTTT 2520

GTGCTAGCGG GCACGCTCCT TGTGCGTGCT GCGGCAGCGA GCGTGTCGTC AAAGGTTGCG 2580

AGGCTTGCCT GGTGATGAAT TGCCACATCC GGCACGCAGC AATCAGGTAG TTTTAGCCCG 2640

CTGCTAGCGC GTAGTTCGGC GAGACGCAGC AGCTCGCCAT CGTCGTACGG CGCAACGACG 2700

CTACCCGCGG AACGCGGATC GTCGAGCATC GATGATCAGG CGCCGCAATA ATTTGGAACG 2760

GGGCTCGCCA GGCCATCGCT GGGCGGCCCG GTCCAATGCC TGAGCTACCT CCGGGGTTTC 2820

GGTTATTTGG TAGCGCGGAC GAGTGGTCGA CATAGAACGA AGTGTGCCAC TTCTAGCAAA 2880

GGTGGTACAC CTACTGGCGG CCGCGGGTTT ACCGCCCCTG CCAGTCACCG CACTTCCGGC 2940

GGCGGACGAC GAGCACCGCG GAATCCACAT GCGGCGGTGG CAGGAACGCG CGCCGTGGCA 3000

GCATGAGGCC GACGGTCAGG GTGAACCTTC GCGCGTTGCG AGAAGCGAAT TTACATACGA 3060

GGGCTCGCTG CAGCACGAGA TCGGCCGCGA CAAGCCCGCT GTTGGGTGCC AGCAGCGTCC 3120

FIGURE 2-5

GCAGCAGGCG GGACGAAATC CCGTACGGCG GGTTCGCCAC AACCCGGAAC GGCCGGCCGG 3180

GCAACCGGAT CGAGGCGGCG TCCGCGTGCA CCACGGTAAT GCCAGGGAAT CGCTCGCGGA 3240

GGACACCGAC TCGTCGCGGG TGCAACTCCA CGGCGACCAC CCGCGCCCCC GCTCGCACTA 3300

GATGCGCCGT CAGTGCCCCT TCGCCGGCGC CGATGTCAAA CACGAGCTCA CCGGACCGCA 3360

CTGCGGCCGC GCTGACTACC CGCGCTGCCC ATTCGTCATG GAGCCGGTGC CAGCCCCATG 3420

CCCGTCGCGA CCGTCCGAGG GCGGACACGA CGTACCGTCA CTGCGTAGAT GCCCACGCGC 3480

CCGACCGTAG CCCGCCACCG GCACTGCGAT CAATCCAATT TCTCGGTTCA GGCAACCTTC 3540

TGGTCATCAC CAGCCCCAGG GCTCTGGCGC CGTCCGCATC AACTCCGAGA TGACGTTGGC 3600

CGTGACGACC CACTAGACCC ACCTGGCAGT AGCCGCATTG TCGCAGTCGG CGAGCCTCAG 3660

TGCGCAGTCG CGTCTAGGTG CAAGGATATT GCCCGTTGAG CAGACAACTC GACGGCGGCG 3720

AGTAAGAACC GGTCAGCCCG CCTCTTAGGC CGCCCGTGGC TGAACCACCG GGGGCAATGA 3780

TGCGATTCCA ATTCGCTGGG CTGAGAACGT AGTGCGTGCC AGATCGTGCA ACGGTGCTAT 3840

TCCATGTGTG CAAGACGGAT TCTCCTGCCG GCAAGTCGAA TTCAAGCTTC CAATCGGTTA 3900

FIGURE 2-6

GCGGCGCCGT GCTCGAGTTT GTGATGGTGA AGCGGGCGAT GAAACCGGTC TGCCACGTCG 3960

ATGTCACCGA CAACGTCGCC CTGGCCGTCG CCGCACTAGC GACCGGGGTG ATGGCGAGTC 4020

CGAGGATGGC AACTATCAAT GCCGACACGG TTGCGTGAAG CGCTGTCCGC CAGCGCCTCA 4080

CGTAAATGTT CAGTCCGGCC ATGACAGCCA ACACTAATGC CAATGAGGCG ATATCGGCCG 4140

TCTCCTCGCG AGCAAGCTAC AGCAACTTTG CTCAACCGCA ACCGTGATGA AATTTGGCCT 4200

CGACCCACCC TGAACCAGAT ATCGGCCCGG CCGAACGCGA ACTTGCGGAC GGGGAAGGCC 4260

AGACAGCCTC GACCCCACTC CCCCGATTAG CGCCGTTCAC CGTTCGCGAC CGGTATCAAC 4320

GGGCTACAGC TCCAACACGA TCCGTAGGGC CGCGTCACGC CGAATGTGCA CTGGTGGCGC 4380

CGACACGCCC GGGCGAGGCC GCCGTCGGCG TGTCAGCTGG TGACTGAGTT GTGCAGACTG 4440

ACCGCGCGCC CTCCTGCCGA ACGGTATGTG CCCATCGACG ATCACGTGGT CCAACCCGCG 4500

TGTGCACACG TGCTGTACTA GGTCACGGTC AGCGAGATTC CCAGCGCAAC CATCATGACC 4560

GCGATCAGGC CGTCGAGGAT TCTCCACGAG CCGGGGTTGG TGAACAGCCC GCGCAACCGG 4620

CCGGCTCCGA ACCCGAGGGT GGCGAACCAT ACCGCACTGG CTGTGACCGC GCCGAGGCCG 4680

FIGURE 2-7

AACAGCCAGC GCTGGTCGCT GTGCTCGTTG GCCAGCGCGC CTAGCAACAC GACGGTGTCG 4740

AGGTAGACGT GTGGGTTGAG GAACGTGAAT GCCGCACAGG TCACCAGGAC CTCGGCTAAG 4800

CGAACCGGCG TGGCGCCAGA TGGGATCAGC GCAACAGGTC GCCACGCCCG CCGGGCCGCA 4860

AGTAGCCCGT AGCCGATTAG GAAGGCGGCG CCGCCAAACT TGACGACATT GAGCGCACGC 4920

GGATGTGCGC CGATCAATGC GCCGAACCCC GCGATACCGG CGGCGATCAG CACGATGTCG 4980

GACACCGTGC ACAGCGCCAC CACCGGCAGC ACGTGCTCAC GCTGGATTCC CTGCCGCAGC 5040

ACGAATGCGT TCTGCGCGCC AATCGCGGCG ATCAGCGTGA AGCAGGCCAG GAAGCCGACG 5100

ACCAGTGGTG AGTTCACGCA ATCGACACTA GGCAGTTTGT ATGGGTCAGT ATAGCTAATA 5160

ATTCTTCATT TACATTAGCA TTATTAATGT GCAGTGCGAC GCTCCGCAGA TGGTCTACAC 5220

CTGAGATGGT GGATCCGCAG CTTGACGGTC CACAGCTGGC CGCATTGGCT GCCGTGGTCG 5280

AACTGGGCAG CTTCGATGCG GCCGCGGAGC GCCTACATGT CACCCCCTCG GCTGTCAGTC 5340

AGCGCATCAA GTCGTTGGAG CAGCAGGTCG GCCAGGTGCT GGTGGTCAGG GAAAAGCCAT 5400

GTCGGGCGAC GACCGCAGGT ATCCCGCTGT TGCGGTTGGC CGCGCAAACA GCGTTGCTCG 5460

FIGURE 2-8

AGTCCGAGGC GCTCGCTGAA ATGGGTGGCA ACGCGTCGCT GAAACGCACG CGGATCACCA 5520

TTGCGGTAAA CGCCGATTCC ATGGCGACAT GGTTTTCGGC CGTGTTCGAC GGTCTCGGCG 5580

ACGTCCTGCT CGACGTTCGG ATCGAGGACC AGGACCATTC CGCGCGGCTG CTACGGGAGG 5640

GTGTGGCGAT GGGCGCGGTG ACCACCGAGC GGAACCCGGT GCCGGGCTGC CGGGTGCACC 5700

CGCTGGGTGA AATGCGCTAC CTACCAGTGG CCAGCAGGCC ATTCGTCCAG CGCCATCTAT 5760

CCGACGGGTT CACTGCCGCC GCGGCGGCTA AAGCTCCGTC ACTGGCGTGG AATCGTGACG 5820

ATGGGCTGCA GGACATGTTG GTGCGTAAGG CCTTTCGTCG CGCCATCACC AGACCGACGC 5880

ACTTTGTCCC GACCACAGAG GGCTTCACCG CCGCAGCGCG CGCCGGGCTG GGATGGGGCA 5940

TGTTCCCCGA GAAGCTGGCA GCATCTCCGC TTGCCGATGG ATCGTTCGTA CGGGTCTGCG 6000

ACATACACCT CGACGTCCCT CTCTATTGGC AATGCTGGAA ACTGGACAGT CCGATCATCG 6060

CGCGAATTAC CGACACGGTG AGGGCGGCGG CAAGCGGTCT GTACCGGGGC CAGCAACGCC 6120

GCCGCCGACC GGGTTGACCG ACGCCAGCAT GTTGTTGTGT CAGCGCGGCT TGGTCTCGAT 6180

GTCCCGGCCT TGCTGGACCC GCTTCCTCAA ACAGGTTGAA CTTAACGACT CAGACGGAAA 6240

FIGURE 2-9


```
CGCTTGAACC GCGACGTCGC TCCGGACACC AATTTGACTC GGCTCTTTGG CAATTGAAGG    6300
TGAGCTGCGA GCAGCCGGGT GACCGCATCG TTGGCCTTGC CATCAATCGC CGGCTCGCGG    6360
ACGTAGATAA TCAGCTCACC GTTGGGACCG ACCTCGACCA GGGGTCCTTT GTGACTGCCG    6420
GGCTTGACGC GGACGACCAC AGAGTCGGTC ATCGCCTAAG GCTACCGTTC TGACCTGGGG    6480
CTGCGTGGGC GCCGACGACG TGAGGCACGT CATGTCTCAG CGGCCCACCG CCACCTCGGT    6540
CGCCGGCAGT ATGTCAGCAT GTGCAGATGA CTCCACGCAG CCTTGTTCGC ATCGTTGGTG    6600
TCGTGGTTGC GACGACCTTG GCGCTGGTGA GCGCACCCGC CGGCGGTCGT GCCGCGCATG    6660
CGGATCCGTG TTCGGACATC GCGGTCGTTT TCGCTCGCGG CACGCATCAG GCTTCTGGTC    6720
TTGGCGACGT CGGTGAGGCG TTCGTCGACT CGCTTACCTC GCAAGTTGGC GGGCGGTCGA    6780
TTGGGGTCTA CGCGGTGAAC TACCCAGCAA GCGACGACTA CCGCGCGAGC GCGTCAAACG    6840
GTTCCGATGA TGCGAGCGCC CACATCCAGC GCACCGTCGC CAGCTGCCCG AACACCAGGA    6900
TTGTGCTTGG TGGCTATTCG CAGGGTGCGA CGGTCATCGA TTTGTCCACC TCGGCGATGC    6960
CGCCCGCGGT GGCAGATCAT GTCGCCGCTG TCGCCCTTTT CGGCGAGCCA TCCAGTGGTT    7020
```

FIGURE 2-10

TCTCCAGCAT GTTGTGGGGC GGCGGGTCGT TGCCGACAAT CGGTCCGCTG TATAGCTCTA 7080

AGACCATAAA CTTGTGTGCT CCCGACGATC CAATATGCAC CGGAGGCGGC AATATTATGG 7140

CGCATGTTTC GTATGTTCAG TCGGGGATGA CAAGCCAGGC GGCGACATTC GCGGCGAACA 7200

GGCTCGATCA CGCCGGATGA TCAAAGACTG TTGTCCCTAT ACCGCTGGGG CTGTAGTCGA 7260

TGTACACCGG CTGGAATCTG AAGGGCAAGA ACCCGGTATT CATCAGGCCG GATGAAATGA 7320

CGGTCGGGCG GTAATCGTTT GTGTTGAACG CGTAGAGCCG ATCACCGCCG GGGCTGGTGT 7380

AGACCTCAAT GTTTGTGTTC GCCGGCAGGG TTCCGGATCC GATGACATAT GACGGGATGG 7440

TTCCCGTTAC CCCACCGGAA TCGATGATCG AGGGGACGGG TATGGGAGTC CCACCATCGA 7500

TCTTTACGTA CAGGGTGGTG ATCGGCGATC CGACGACCTC GACGTTGGGC GCAGGTAGCG 7560

GGTTGGGACC GAACACGAGC TCACCTGCGG GTGCGTCGAT GAGCACTCCC TGGTTGAGGT 7620

CACCCGGTAA CGCCATCGTC GGAATGCTGG GGCCTGGTCC CACCGCATTG GACCCAACTC 7680

CCAGAACGCC GTCCACGCCG ACGGCACCGA AATAGGCTTC GAACGGGGTT GTTGTCGGAT 7740

CGGCCAGCAA GGCGCTGAAG TAGGTCGAAA TGGCGAAGGG GGACGTTGGG ATGGACAAGA 7800

FIGURE 2-11

GGACGACATT AACGGTGGTC GGCGCGGTGA CGATGCCATT CCCGAAGTCC ACCGTCGTGG 7860

TATACGTGGC GAAGATGTAG TACAGCCCCC CGCTGTAACC GCTGATGCTC AATCCGGTTG 7920

GGAGGCCCAT GTGAAGCACT CCCAGGATTC CCCCGACATC CTCAGGTGAG ACAACAAGAT 7980

CAGCGGATCC GGTGTCGACC AGAATGGTTG ACGTCGGTCC GCCGTTGACG TTGGCATGTA 8040

CCGTCGGCTC TGTGACATGA ATTATCTCCA GCGGGACGGT TCTGCCGTCG CCGCCTGGCC 8100

CACTGACGCC GTATCCGCCA TACAACAGGC CGCCGCGGCC TCCGGCGCCG CCCGTGCCAC 8160

GATCGACACC GACTCCATCA CCGAGGCCGC CGGCTCCGCC GTTCGCTCCC CAGCCCAAAA 8220

GTCCTGTGGC ACCGCCGGTT CCGCCGGCTC CACCACTCAT GCCGGGGATG CTCGACGCGC 8280

CCCCGGGCCC GCCGATCCCG CCGTTGCCGA GCAGCCAGCC ACCGTTTGCC GCCGGCTCCC 8340

CCGGGGGCGT TGGGGCCGCC GGCTCCCCCG GCGCCGCCAT TGCCGATCAA CGCCGCGGAA 8400

CCGCCGGCAC CGCCGCCGAC CCCCGACGCC GTCGCGGAAT AACCGTTGCC GCCGTTGCCG 8460

TACAGCAGCC CACCCGCCCC GCCATTCGGA CTCGTTGCCG TCCCGGGTGC TCCGTCGCCG 8520

ATTAGCGGAC GCCCCAACAA CGTTTCGGTT GGTGCATTGA CCGCGCCCAG CAGATCGTGC 8580

FIGURE 2-12

TGCGCGGTCT GCAACTGTGA CGCGATGGTG GCCTCCGCGG CCGCATACGA TCCTGACGCC 8640

GAGTTCAGCG TCTGCACGAA CTGTTGATGG AAAGCGCTCG CCTGCGCGCT GACCGCTTGA 8700

TATTCCTGAC CGAACCTGGC AAACAGCGCT GCCACCGCCG CCGATACCTC ATCAGCGCCA 8760

GCGGCCGCAA GCGCGGTGGT CGAGGCGGCA GCCGCGGCAT TCGCCGCGCG CAGTGTGGAA 8820

CCTATGTTCT CCACATCCGC TGCCGCGGAC GTCAAGAACT CGGGAACCAC GACCAGAAAT 8880

GACACGCCGC CCCTCCGCCT CGATCACCAT CCCTGCGCGC ATACAGCGTA TCCAGACGCT 8940

GCCTTTGACA TCTCGGATTT TCAGTAGCTA CCGCACGGCA CAGCACGCGT TAGGTAGATA 9000

GTGGCTATTT GCTGGTACCA TCTACCTGTG GCGCTGAATA TCAAAGACCC TGAGGTAGAC 9060

CGACTAGCCG CCGAACTCGC TGACCGGCTG CACACCAGCA AGACTGCCGC CATCCGGCAT 9120

GCCCTGTCTG CCCAGCTGGC GTTTTTGGAG TCGCGCGCCG GCGACCGTGA GGCACAACTT 9180

CTCGACATCT TGCGTACCGA AATCTGGCCC CTGCTTGCCG ACCGCTCCCC CATCACCAAG 9240

CTCGAGCGCG AACAAATCCT CGGCTACGAC CCCGCAACCG GAGTCTGAGC ACCGCAATGA 9300

TCGTGGACAC AAGCGCCGTG GTGGCCCTGG TTCAAGGCGA GCGGCCGCAC GCCACCCTGG 9360

FIGURE 2-13

TCGCGGCCGC CCTGGCCGGC GCCCATAGCC CCGTCATGTC TGCACCCACC GTCGCCGAAT 9420

GCCTGATTGT CTTGACCGCC CGTCACGGCC CCGTTGCGCG CACGATCTTC GAACGACTTC 9480

GCAGCGAAAT CGGCTTGAGC GTGTCATCTT TCACCGCCGA GCATGCCGCT GCCACGCAAC 9540

GAGCCTTTCT GCGATACGGC AAGGGGCGCC ACCGCGCGGC TCTCAACTTC GGAGACTGTA 9600

TGACGTACGC GACCGCCCAG CTGGGCCACC AACCACTGCT GGCCGTCGGC AACGACTTCC 9660

CGCAAACCGA CCTTGAGTTC CGCGGCGTCG TCGGCTACTG GCCAGGCGTC GCGTAACCGT 9720

ATGCGCGGTG ATCGCTGTTT GTAATGAGTT CAGCGACACG AAGAATAAAA TATGGGTAGC 9780

CGAAATCACT AAGCTACAGT GCTGGTGCAC GCCATGAAAG ACCGTCAATG ACAAGGAGGA 9840

CGGCCGAAAT GCCCAAGGAC CGACTGCCGG ACTTGACGCC CACAGGAGCG TACGCACCGG 9900

CCAACAGCGG CATGACCATG GCAAGGCAGG ACGGCCCTCG ATGACCGGCA AGCGCGTTGA 9960

GCGGGTGCAC GCAATCAATT GGAACCGGTT GCTCGATGCT AAAGATTTGC AGGTCTGGGA 10020

ACGTTTGACC GGTAACTTTT GGTTGCCGGA AAAGATTCCG CTCTCCAACG ACCTGGCATC 10080

TTGGCAAACG TTGAGTTCCA CCGAGCAGCA GACGACGATC CGGGTGTTCA CCGGCTTGAC 10140

FIGURE 2-14

CCTGCTCGAC ACCGCGCAGG CGACGGTGGG AGCAGTGGCC ATGATCGACG ACGCGGTCAC 10200

CCCCCACGAA GAGGCGGTCC TGACCAACAT GGCGTTCATG GAGTCAGTGC ACGCCAAGAG 10260

CTACAGCTCG ATCTTCTCGA CCCTGTGCTC GACCAAGCAG ATCGACGATG CCTTCGACTG 10320

GTCGGAACAG AACCCTTACC TGCAGCGAAA AGCGCAGATC ATCGTCGACT ACTACCGCGG 10380

TGACGACGCG CTCAAGCGCA AAGCATCGTC GGTAATGCTG GAGTCCTTCC TGTTCTACTC 10440

CGGCTTCTAC CTGCCCATGT ACTGGTCGTC GCGGGGTAAG CTCACCAACA CCGCCGATCT 10500

GATCCGGCTG ATCATCCGAG ATGAAGCCGT CCACGGCTAC TACATCGGCT ACAAATGTCA 10560

ACGAGGTTTG GCCGACCTGA CCGACGCCGA GCGGGCCGAC CACCGCGAAT ACACCTGCGA 10620

GCTGCTGCAC ACGCTCTACG CGAACGAGAT CGACTATGCG CACGACTTGT ACGACGAGTT 10680

GGGCTGGACC GACGACGTTT TGCCCTACAT GCGTTACAAC GCCAACAAGG CGCTAGCCAA 10740

CCTGGGATAC CAGCCTGCAT TCGATCGTGA CACCTGCCAG GTGAACCCGG CCGTGCGCGC 10800

AGCTCTCGAC CCCGGTGCAG GGGAGAACCA CGACTTTTTC TCCGGCTCCG GAAGCTCATA 10860

CGTAATGGGC ACCCACCAAC CCACCACCGA CACCGACTGG GACTTCTAAC CGCCCAGCGC 10920

FIGURE 2-15

GTCGGGGGCG TCGAGCACCA CGCGACACCG GGCCCGATCG ATCTGCTAGC TTGAGTCTGG 10980

TCAGGCATCG TCGTCAGCAG CGCGATGCCC TATGTTTGTC GTCGACTCAG ATATCGCGGC 11040

AATCCAATCT CCCGCCTGCG GCCGGCGGTG CTGCAAACTA CTCCCGGAGG AATTTCGACG 11100

TGCGCATCAA GATCTTCATG CTGGTCACGG CTGTCGTTTT GCTCTGTTGT TCGGGTGTGG 11160

CCACGGCCGC GCCCAAGACC TACTGCGAGG AGTTGAAAGG CACCGATACC GGCCAGGCGT 11220

GCCAGATTCA AATGTCCGAC CCGGCCTACA ACATCAACAT CAGCCTGCCC AGTTACTACC 11280

CCGACCAGAA GTCGCTGGAA AATTACATCG CCCAGACGCG CGACAAGTTC CTCAGCGCGG 11340

CCACATCGTC CACTCCACGC GAAGCCCCCT ACGAATTGAA TATCACCTCG GCCACATACC 11400

AGTCCGCGAT ACCGCCGCGT GGTACGCAGG CCGTGGTGCT CAAGGTCTAC CAGAACGCCG 11460

GCGGCACGCA CCCAACGACC ACGTACAAGG CCTTCGATTG GGACCAGGCC TATCGCAAGC 11520

CAATCACCTA TGACACGCTG TGGCAGGCTG ACACCGATCC GCTGCCAGTC GTCTTCCCCA 11580

TTGTGCAAGG TGAACTGAGC AAGCAGACCG GACAACAGGT ATCGATAGCG CCGAATGCCG 11640

GCTTGGACCC GGTGAATTAT CAGAACTTCG CAGTCACGAA CGACGGGGTG ATTTTCTTCT 11700

FIGURE 2-16

TCAACCCGGG GGAGTTGCTG CCCGAAGCAG CCGGCCCAAC CCAGGTATTG GTCCCACGTT 11760

CCGCGATCGA CTCGATGCTG GCCTAGACTC GCGAGGACCG CGCGGTGGTC ACTGCGCGGA 11820

TTTGGGGCGG CGGAAGTGAG TGTTCGGTGC GCCCACTGCG GTGACTCACC TGCAGCGCCG 11880

GCATCGACAG GCCGGGAGCT CAAGAATCGT CGCTAGAGAA TCTATGGTGC GTTAGAGGAT 11940

TCCCTGCTAG ACAGCCTTGG TGCGGTGGTC GGCCCGCGGA CGAGAGGATA TGCGATCCAC 12000

AAGCTGGGTT TCTGCAGCGT CGTCATGCTC GGGATCAACT CGATAATCGG CGCCGGTATC 12060

TTCCTAACTC CAGGTGAGGT GATCGGGCTC GCAGGACCCT TCGCGCCGAT GGCCTATGTT 12120

TTAGCTGGCA TTTTCGCGGG TGTCGTGGCG ATCGTCTTCG CGACGGCGGC AAGGTACGTC 12180

AGAACAAACG GTGCCTCCTA CGCCTACACA ACGGCCGCAT TTGGGCGCCG GATCGGCATC 12240

TATGTCGGTG TCACCCACGC CATTACCGCG TCCATCGCTT GGGGGGTGTT GGCTTCTTTT 12300

TTCGTCTCGA CGCTGTTGCG AGTGGCCTTC CCCGACAAGG CCTGGGCCGA CGCCGAGCAA 12360

CTGTTCAGTG TGAAGACGCT GACGTTTCTC GGCTTTATCG GCGTGCTGTT GGCCATCAAC 12420

CTCTTCGGCA ACCGGGCGAT CAAGTGGGCC AACGGAACGT CAACGGTAGG CAAGGCATTC 12480

FIGURE 2-17

GCGCTCTCGG CATTCATTGT CGGCGGGCTG TGGATCATCA CCACCCAGCA CGTGAACAAC 12540

TACGCAACGG CGTGGTCGGC ATACAGCGCG ACCCCGTACT CGTTGCTTGG CGTCGCCGAA 12600

ATTGGCAAGG GCACGTTCTC GAGTATGGCG CTGGCCACGA TTGTCGCGTT GTACGCATTC 12660

ACCGGTTTCG AATCGATCGC GAACGCCGCC GAAGAAATGG ACGCGCCGGA CCGGAACCTG 12720

CCGAGAGCTA TACCGATCGC GATCTTCTCG GTTGGCGCGA TCTACTTGCT CACCCTAACG 12780

GTAGCGATGC TGCTCGGATC GAACAAGATC GCCGCGTCGG GCGACACCGT GAAACTGGCC 12840

GCGGCCATCG GAAACGCTAC CTTCCGAACG ATCATCGTCG TCGGAGCCCT GATATCGATG 12900

TTCGGCATCA ATGTCGCGGC CTCGTTCGGT GCACCGCGGC TTTGGACCGC GTTAGCGGAC 12960

AGCGGGGTTC TGCCGACACG CTTGTCACGC AAGAACCAAT ACGACGTGCC GATGGTCTCC 13020

TTCGCAATTA CGGCGTCGTT GGCGCTCGCA TTCCCGTTGG CGCTGCGGTT CGACAACCTG 13080

CACCTGACCG GCCTGGCGGT GATCGCCCGA TTCGTCCAGT TCATCATCGT GCCGATCGCT 13140

CTCATCGCAT TGGCGAGGTC TCAGGCAGTA GAACATGCTG CTGTGCGGCG AAATGCGTTC 13200

ACCGACAAGG TGTTACCGCT TGTTGCGATC GTGGTCTCGG TTGGGCTGGC AGTGTCCTAC 13260

FIGURE 2-18

GACTACCGCT GCATCTTTCT AGTGCGGGGT GGTCCGAACT ACTTCTCGAT TGCTTTGATC 13320

GTGATCACGT TCATCGTGGT ACCGGCGATG GCTTATCTGC ACTACTACCG AATCATTCGC 13380

CGGGTTGGCG ATCGGCCGAG CACTCGCTAG ATTCCGTTGG CGCTGAGCTC GAACGGGAGA 13440

ACACAACGGC GAGCGATGGC GGGAATAGCC TGGTCGGTGC GGGCAAGATT TCAACCTGCA 13500

TTCCCGGATC GGCGGCGCGG GCAAGCGTCT GCAACGCCGA GGGACTGTAG GCACGTAGTG 13560

CGCTGATAAA GCCGTCGTGC ATGCTCGAGC GCATCGACGA CCATGGCAGC AGCAGTAGGT 13620

GGAGCGGCAG TAGCAGCACC GAAGAGAGCG TGAACGACAG CGGTTTCTGC CGTTTGAGGT 13680

CGATGATCAG AAAGCGCTTC CCCACCCGGG TGGCCTCGGC GATCGCTTTG CAGGCGACCG 13740

TAGGCGGCAG GTGGTGAAAT GCCAGCGCGA AGACCGCCAG GTCATAGCTG TGGTCGTGGC 13800

CGTCGATTGC GGTGGCGTCG ATCACTTGGG TGCGTGCTCG CGGATGTGTT CCCAGCTCTC 13860

CCGCGGCGAT GTTGGCCACC GAGGTGGGAT CTAGATCGCT GATCGTCACC GTCGCTGTCG 13920

GGTGTAGCTC GAGGATTTTC GCTGAGAGCT TGCCATGGCC CGCACCAAGT TCCAGGATTC 13980

GCGGGTTGGG AATGTCAGAA ACAAGTTTCA GGGCTATCCG GGCGTACTTC TCGTGCAGGT 14040

FIGURE 2-19


```
TGGTCAGGGT GCCCACCCGG TCGAGCACCC CGATGATCTT CTGTTTGACC TCATCGGGCA   14100
CATCGTCGCG GTCGAGGTAC TCCAGTGCGT CGGTCTGGAA TCGACGATCC AGCCAAGACG   14160
CGTCGGGGCC ACCCCGTGGC ATCGTGGCGA TCGCCTGCTC GCGGATGTTC GCCTCACCCA   14220
TGGCAGCTCT TCCCCTCTCG ACGTCCCGTG TTCGCAATGC TATGAGACCG CTGACCGGGC   14280
TCCCCAGCCC GCCGGTCGCG CGTGCTTAGC TACGTAGCAG AGGGGCCGTC ACTTCGAGGG   14340
CTGCCGCCAC TCGGTGATCT TGCGGCCCAA TGAATCGGCC GCGTTCGAGG CTGCCCGTCC   14400
CACGGCTTTG GTTCACGGTG AAGATCGCAC AGCCGGTGCC GGAAAAGTCC GCGGCACCGA   14460
TGTCGGTCAG CAAGACGTTG AAGAGAAACC CCGAGATCAC CGCCCATGGG ATCGTCATCA   14520
ACACCCCAGG CAGCGTCGAC ACCCGCGCCA CGAACCAGCA CTGAAGTAGG TATTCACGCC   14580
ACGCGAAAGG CGGCTTGAAC ATGCACACGG ACGTGTCGAG CGTCATCGCG AAGAAATCGC   14640
CCAGCGCGCC CACCGGCCGC AAGACCGGAT CAGCGACCCG ACCGGCCGCC TTGTCGGCCA   14700
CGATTACCAT GGCGCGGCGC ACCAGCTGGA TTCGATGCTG GGCCGTTGGG TGAGGTGGCG   14760
CACGCTGGCC CCCCGGACAG GTCGACGATC GGTGACATTG GTGAGCGTAC GCGGCAGAGA   14820
```

FIGURE 2-20


```
CCGCTGATGT CCATAGCCAA TACGCGATTG CTTGGACAAC TGATCGGTAA ATAGCAATGC   14880

AAACTGGCAT ATATTGGCTA TGATGTATCT TGCTAGTATC CTATAGCGCG GGGCGATGTG   14940

CTCTGCTGCC TTGGCGGCCG ACAGGCGCAT CACCGGTCAA GCCGTTGGCT CGAGTCACGC   15000

TGGCGAGGCA CCACGATCAG GCATCAACAG CGCGCCCGAC GGGCGGTGAT CGGATGCCGC   15060

ATCCTGACCG CCTCGATTCG GGCCCGCCGA CCAGAGCCTT CGCGACCGGC GAGGTTGCCA   15120

CCATGGTCGT CGAAGCAACT TGCTGCTAAC GAGCCTGTAG TTTTGCCAGC CCCCACTCGC   15180

GCTTTGTCTG CAGGTTTTCA GGCTCAGCGA CGGCTCATGT CGTTGCGCAC GGCGAATTC    15239
```

FIGURE 3-1


```
GAATTCACTT AGCTAACACC AGTTCTAGCA GCTGTCGGCG CGACTTCTTG TCAGTGCCCG      60
ACGTTATGAT TCGAACATGT TAGCGAATAG CCGGGAGGAG CTTGTCGAGG TCTTTGATGC     120
GCTGGATGCC GAGCTGGACC GCTTGGACGA GGTGTCTTTT GAGGTGTTGA CCACCCCGGA     180
ACGGCTGCGG TCTCTGGAAC GTCTGGAATG CTTGGTGCGC CGGCTACCGG CGGTCGGGCA     240
CACGTTGATC AACCAACTCG ACACCCAAGC CAGCGAGGAA GAACTGGGCG GCACGCTGTG     300
CTGCGCGCTG GCCAACCGGT TACGCATCAC CAAGCCCGAC GCCGCCCTAC GCATCGCCGA     360
CGCCGCCGAT CTCGGACCTC GTCCGAGCAC TCACCGGCGA ACCGCTAGCC CCACAGTTTG     420
ACCGCCACCG CCACCGCCCA ACGCCAGGGC CTGATCGGCG AAGGCGCACA TCAAAGTGAT     480
TCGCGCCCTT TTTCGGCCCA ACCTGCCCGC CGCGGTGGAT GTGTCCAAAC CCGCCAGGCC     540
GCCGAAGCCC GACCTGGCCG CAAACCGCTC AAATATCGTC CCGACGAGCT GGCCCGCTAC     600
GCCCAGCGGG TCATGGACTG GCTACACCCC GACGGCGACC TCACCGACAC CGAACGCGCC     660
CGCAAACGCG GCATCACCCT GAGCAACCAG CAATACGACG GCATGTCACG GCTAAGTGGC     720
TACCTGACCC CCCAAGCGCG GGCCACCTTT GAAGCCGTGC TAGCCAAACT GGCCGCCCCC     780
```

FIGURE 3-2

GGCGCGACCA ACCCCGACGA CCACACCCCG GTCATCGACA CCACCCCCGA TGCGGCCGCC 840

ATCGACCGCG ACACCCGCAG CCAAGCCCAA CGCAACCACG ACGGGCTGCT GGCCGGGCTG 900

CGCGCGCTGA TCGCCTCCGG GGAACTGGGC CAACACAACG GTCTTCCCGT CTCGATCGTG 960

GTCACCACCA CCCTGACCGA CCTGCAAACC GGCGCCGGCA AGGGCTTCAC CGGCGGCGGC 1020

ACCCTGCTAC CCATGGCCGA TGTGATCCGC ATGACCAGCC ACGCCCACCA CTACTCCCCC 1080

GCAAGCGGGA GGTACCCCCA GGCGATCTTC GACCACGGCA CACCCCTGGC GCTGTATCAC 1140

ACCAAACGCC TAGCCTCCCC GGCCCAGCGG ATCATGCTGT TCGCCAACGA CCGCGGCTGC 1200

ACCAAACCCG GCTGTGACGC ACCGGCCTAC CACAGCCAAG CCCACCACGT CACCGGCTGG 1260

ACCAGCACCG GACGCACCGA CATCACCGAC CTCACCCTGG CCTGCGACCC CGACAACCGA 1320

CTCGCCGAAA AAGGCTGGAC CACCCGCAAA AACACCCACG GCCACACCGA ATGGCTACCA 1380

CCACCCCACC TCGACCACGG CCAACCGTGG ACCTGTGAGA TACACTACAC CTGTGCGTGC 1440

TGCTGTCTAC CTCCGAATCT CAGAAGACCG CTCCGGCGAA CAGCTCGGCG TGGCCCGCCA 1500

ACGCGAGGAC TGCCTAAAGC TGTGCGGGCA GCGAAAATGG GTGCCCGTCG AGTACCTCGA 1560

FIGURE 3-3


```
CAACGACGTC AGCGCATCAA CCGGCAAGCG CCGCCCCGCC TACGAGCAGA TGTTGGCCGA    1620

CATCACCGCC GGCAAGATCG CCGCCGTGGT GGCCTGGGAC CTGGACCGGC TCCATCGCCG    1680

TCCCATCGAG CTGGAAGCCT TCATGTCATT AGCCGACGAG AAGCGGCTGG CCCTGGCCAC    1740

CGTCGCCGGC GACGTTGACC TGGCGACACC CCAGGGCCGG CTAGTCGCCC GCCTGAAGGG    1800

GTCGGTGGCC GCTCACGAAA CCGAGCACAA GAAGGCACGA CAGCGCCGCG CCGCCCGCCA    1860

GAAAGCTGAA CGCGGCCACC CCAACTGGTC GAAAGCCTTC GGCTACCTGC CCGGCCCCAA    1920

CGGTCCCGAA CCCGACCCCC GGACAGCGCC GCTGGTCAAA CAGGCCTACG CCGACATCCT    1980

CGCCGGGGCG TCCCTGGGCG ACGTGTGCCG CCAGTGGAAC GACGCCGGGG CGTTCACCAT    2040

CACCGGCCGC CCGTGGACGA CTACAACGCT GTCGAAATTC TTGCGCAAAC CCCGCAACGC    2100

CGGACTACGC GCATATAAGG GTGCCCGCTA CGGCCCGGTG GACCGCGACG CGATTGTCGG    2160

CAAGGCCCAG TGGTCGCCGC TGGTGGACGA GGCGACGTTC TGGGCCGCCC AGGCCGTGCT    2220

GGACGCCCCC GGCCGCGCCC CCGGCCGCAA AAGCGTGCGC CGCCACCTGC TGACCGGGCT    2280

GGCAGGCTGC GGCAAATGCG GCAACCACCT GGCCGGCAGC TACCGCACCG ACGGCCAGGT    2340
```

FIGURE 3-4

CGTCTACGTG TGCAAGGCGT GCCACGGGGT GGCCATCCTG GCCGACAACA TCGAACCGAT 2400

CCTGTATCAC ATCGTGGCCG AGCGGCTGGC CATGCCCGAC GCCGTTGACT TGTTGCGCCG 2460

GGAGATTCAC GACGCCGCCG AAGCCGAAAC CATCCGCCTG GAACTGGAAA CCCTCTACGG 2520

GAGCTGGACA GGCTCGCCGT CGAACGCGCC GAAGGGCTAC TGACCGCGCG CCAGGTGAAG 2580

ATCAGCACCG ACATCGTCAA CGCCAAGATA ACGAAACTTC AGGCCCGCCA ACAGGATCAG 2640

GAACGGCTCC GAGTGTTCGA CGGGATACCG TTGGGAACAC CGCAAGTCGC CGGGATGATA 2700

GCCGAGCTGT CGCCGGACCG GTTCCGCGCC GTCCTCGACG TCCTCGCTGA AGTCGTTGTC 2760

CAGCCGGTCG GCAAGAGCGG CAGGATATTC AATCCCGAAC GGGTGCAGGT GAATTGGCGA 2820

TGAGCCGGCA CCACAACATC GTGATCGTCT GTGACCACGG CCGCAAAGGC GATGGCCGCA 2880

TCGAACACGA GCGCTGCGAT CTTGTCGCGC CGATCATTTG GGTCGACGAG ACCCAGGGCT 2940

GGTTACCGCA GGCGCCAGCG GTGGCAACAT TACTCGACGA CGACAACCAG CCGCGAGCCG 3000

TTATTGGCTT GCCGCCCAAC GAGTCTCGCC TACGACCTGA AATGCGCCGC GACGGGTGGG 3060

TGCGGCTGCA CTGGGAATTC GCCTGCCTGA GGTACGGCGC CGCCGGCGTG CGCACGTGCG 3120

FIGURE 3-5

AGCAGCGGCC CGTGCGGGTT CGCAACGGCG ACCTGCAAAC ACTGTGCGAG AACGTTCCGC 3180

GGCTACTGAC CGGACTGGCC GGCAACCCCG ACTACGCACC GGGTTTTGCG GTGCAGTCGG 3240

ACGCGGTGGT CGTCGCCATG TGGCTGTGGC GCACGCTCTG CGAAAGCGAC ACGCCGAACA 3300

AACTACGCGC CACCCCAACG CGTGGTAGCT GCTAGACTCC GACGTAGCCG GCTTCGACTC 3360

CGGGGTTTTG GTGTCCCCAA GGAGTCGCAC GTGTCGACCA TCTACCATCA TCGCGGCCGC 3420

GTAGCCGCAC TGTCTCGTTC CCGCGCATCC GACGATCCCG AGTTCATCGC CGCGAAAACC 3480

GATCTCGTTG CCGCGAACAT CGCGGACTAC CTCATCCGCA CCCTCGCCGC AGCGCCGCCC 3540

CTGACTGACG AGCAGCGCAC CCGGCTGGCC GAGCTGCTGC GCCCCGTGCG GCGGTCAGGC 3600

GGTGCCCGAT GACCGCCGGC GCCGGCGGGT CGCCGCCGAC GCGACGATGC TCGGCCACGG 3660

AGGACCGGGC ACCCGCGACA GTCGCCACAC CGTCTAGCGC CGATCCTACC GCGTCACGCG 3720

CCGTGTCGTG GTGGTCGGTG CACGAGCATG TCGCGCCGGT CCTGGATGCT GCCGGGTCGT 3780

GGCCGATGGC CGGCACACCG GCCTGGCGTC AGCTCGACGA CGCCGATCCT CGCAAATGGG 3840

CCGCGATCTG CGACGCAGCC CGGCACTGGG CTCTGAGGGT AGAGACGTGC CAGGAGGCGA 3900

FIGURE 3-6

TGGCGCAGGC GTCACGTGAC GTATCTGCGG CCGCCGACTG GCCCGGCATC GCCCGCGAGA 3960

TCGTCCGACG GCGCGGCGTG TACATCCCGC GGGCGGGGGT GGCGTGATGG CCGACATCCC 4020

CTACGGCACC GACTATCCCG ACGCCCCCTG GATCGACCGG GACGGGCACG TGCTCATCGA 4080

CGACGGTGGC AAACCGACGC AAGTTCATCG CGGCCAAGCC CGAATCGCCT ACCGGCTAGC 4140

CGAACGTTAC CAGGACAAGC TGCTGCACGT GGCCGGGATC GGCTGGCACT CCTGGGACGG 4200

CAGACGCTGG GCAGCCGACG ACCGCGGCGA AGCCAAACGT GCAGTGCTGG CAGAGCTGCG 4260

CCAAGCGCTC TCAGACAGCC TCAACGACAA GGAATTACGC GCCGACGTCC GAAAATGCGA 4320

ATCGGCGTCC GGCGTGGCCG GCGTGCTCGA CCTGGCCGCC GCACTGGTAC CATTCGCCGC 4380

GACGCTAGCC GACCTCGACA GCGACCCGCA CTTGCTCAAC GTCGCGAATG GGACGCTGGA 4440

CCTGCACACG CTCAAATTGC GGCCCCACGC GCCCGCTGAC CGCATCACAA AGATATGCCG 4500

CGGTGCCTAC CAGTCCGACA CCGAATCGCC TCTCTGGCAA GCGTTCTTGA CCCGCGTTCT 4560

GCCCGATGAA GGTGTGCGCG GGTTCGTGCA ACGCCTGGCC GGCGTCGGCC TACTAGGCAC 4620

CGTCCGCGAA CATGTCCTGG CGATTCTTAT CGGTGTAGGT GCCAACGGAA AATCTGTGTT 4680

FIGURE 3-7


```
CGACAAGGCG ATTCGCTATG CCCTTGGCGA TTATGCCTGC ACCGCTGAGC CTGACCTTTT    4740

CATGCACCGG GAAAACGCTC ACCCAACAGG CGAAATGGAC CTCCGCGGCG TGCGATGGGT    4800

AGCGGTATCC GAGAGCGAAA AAGATCGCCG GCTGGCCGAA TCAACGATAA AACGGCTGAC    4860

TGGCGGCGAC GCCATCCGCG CCCGAAAGAT GCGGCAAGAC TTCGTGGAAT TCGAGTGGTG    4920

CCGTTTGAAG TAGTGATTCC TGCCGACGAG CAGGACCGGG AACTGGACGC ACGGTTGCAG    4980

TTGGAGGCCG ACAGCATCCT GTCCTGGGCG GTGGCCGGAT GGAGCGACTA TCAGCGAATC    5040

GGACTATCCC AGCCGGACGC GGTGCTCGCG GCAACGTCGA ATTACCGCGA GGACTCCGAC    5100

ACGATAAAGA GGTTCATCGA CGACGAATGC GTCACCAGCT CGCCGGTGCT GAAAGCCACT    5160

ACTACGCATC TGTTCGAGGC GTGGCAAAGG TGGCGGGTGC AAGAAGGCGT ACCCGAAATC    5220

TCGCGCAAAG CGTTCGGCCA GTCGCTCGAC ACCCACGGAT ACCCGGTCAC TGACAAGGCC    5280

CGTGATGGTC GTTGGCGGGC CGGAATAGCG GTGAGAGGGG CCGATGATTT CGATGATTAG    5340

CACACCTAAC GTGACGCATG TGACGCATTT CCAGGTTCGC CTACGCGCGC GCACGTATGG    5400

CGGTTATACC GCGCAAACGT CACATGCGTC ACGGCCTGCC GTGCCGTTCT GCCCAGGATG    5460
```

FIGURE 3-8


```
CGGTACCTAC CTGGCCGTTC ACGGCCGCCA CCGGGCGGAC TGTACCGCCA AACCAGCAAA    5520
CACCGGCGGT GCCGCATGAC CGCTGTCGCG ATCACCCCGG CATCCGGCGG TCGGCACAGC    5580
GTCCGATTCG CCTACGACTC TGCGATCGTG TCGTTGATCA AGTCCTCGAT CCCCGCCTAT    5640
GCCCGCTCCT GGTCCGCGCA CACCCGCTGC TGGTTCATCG ACGCTGACTG GACCCCACTG    5700
CTGGCCGCCG AGCTGCGCTA CCACGGCCAC ACCGTCACCG GACCCGCCGA CCCGGCGCAA    5760
CAGCAGTGCA CCGACTGGGC CAAAGCGTTG TTCCGGGCGG TCGGACCCCA GCGGACACCC    5820
GCCGTGTACA GGGCTTTATC CAAAGTGCTG CACCCCGACG CCCCAACCGG ATGCCCGATA    5880
CTGCAACAGC AGCTCAATGC CGCCAGAACC GCACTTACCA ACCCTGCTTG AAAGGACACA    5940
AGCCATGGCT GAAACCCCCG ACCACGCCGA ACTGCGGCGA CGAATCGCCG ACATGGCTTT    6000
CAACGCCGAT GTCGGTATGG CGACCTGCAA ACGCTGTGGT GACGCCGTGC CGTACATCAT    6060
CCTGCCGAAC CTGCAGACCG GCGAACCCGT CATGGGTGTC GCCGACAACA AATGGAAGCG    6120
CGCGAACTGT CCCGTCGACG TCGGTAAGCC GTGCCCGTTC CTAATCGCCG AGGGTGTCGC    6180
CGACAGTACC GACGACACCA TAGAGGTCGA CCAGTGACCC CGATCAACCG GCCCCTGACC    6240
```

FIGURE 3-9

AACGACGAAC GACAACTGAT GCACGAGCTG GCAGTCCAGG TTGTCTGCTC GCAGACGGGT 6300

TGCTCACCCG ATGCGGCGGT CGAAGCACTC GAATCCTTCG CGAAAGACGG AACACTTATC 6360

CTCCGCGGCG ACACCGAGAA CGCCTACCTC GAAGCCGGAG GCAATGTTCT TGTCCATGCC 6420

GATCGTGACT GGCTTGCCTT CCACGCGTCG TATCCCGGCA ACGACCCGCT GCGAGACGCC 6480

CGACCTATCG AGCAGGACGA CGACCAGGGG GCGGGGTCGC CATCGTGACC AGGCCCAGCC 6540

CGGACACCGC CACGGTGCCG GCGCGCATGC ACGCTCATTA CCTAGACTAA AAATTGATGG 6600

GAGGACCGAT GCCAAGACCA CCGAAACCGG CCCGGCTCAA ACTGGTTGAG GGCCGCTCCC 6660

CCGGCCGCGA TTCCGGCGGC CGGAAAGTCC CCGAGTCGCC GAAGTTTATC CGTCAGGCAC 6720

CGGATGCCCC GGACTGGCTC GACGCCGAGG CGCTGGCCGA ATGGCGGCGC GTCGCACCGA 6780

CTTTGGAGCG GCTTGACCTG CTCAAACCTG AGGATCGGGC GCTCCTGTCC GCGTACTGCG 6840

AGACCTGGTC CGTCTACGTC GCGGCGGTTC AGCGGGTCCG CGCCGAAGGC CTCACAATTA 6900

CCTCACCGAA ATCCGGTGTC GTGCACCGGA ACCCGGCGGT GACGGTTGCG GAGACGGCGC 6960

GCATGCATCT GCTGCGCTTG GCCTCCGAGT TTGGCCTGAC CCCGGCCGCC GAGCAGCGAC 7020

FIGURE 3-10

TGGCGGTGGC GCCGGGCGAC GACGGCGACG GGCTCAACCC GTTTGCCCCG GACCGGTGAT 7080

GACCTTTTGT GTGTGATACA ATCGAGTTTG GCATCTCGGC ATCCGCTGAC GCCGGGCAGT 7140

CGCCGCGGGG CGGCTGGAAC CCGGATAGCG GCCGCCATGC GCCACAAGCG ATTCCGCGCG 7200

TTTCTTGCGT CTGCTAGGTG GTGGCCGAAT TTTGAGTAGC ATCCTTTTCC GCATGGCCGA 7260

GCTGCGGTCT GGCGAAGGCC GAACCGTGCA CGGCACCATC GTGCCCTACA ACGAGGCGAC 7320

CACCGTCCGC GACTTCGACG GCGAGTTCCA GGAAATGTTC GCTCCTGGCG CTTTTCGGCG 7380

CTCCATCGCC GAGCGCGGCC ACAAATTGAA GCTGCTGGTC TCTCACGACG CTCGAACCCG 7440

CTACCCGGTG GGCCGGGCCG TTGAGTTGCG GGAGGAGCCT CACGGCTTGT TCGGGGCGTT 7500

CGAGATTGCG GACACCCCGG ACGGCGACGA GGCTTTGGCG AACGTAAAAG CTGGTGTCGT 7560

CGACTCGTTT TCGGTGGGTT TCCGACCGAT CCGGGACCGT CGCGAAGGGG ATGTGCTGGT 7620

GCGCGTCGAA GCGGCGCTGT TAGAGGTTTC CCTAACCGGC GTTCCGGCCT ATTCGGGGGC 7680

ACAAATCGCC GGGGTGCGCG CGGAATCGCT TACAGTCGTT TCCCGTTCGA CAGCCGAAGC 7740

CTGGCTGTCC CTACTCGATT GGTGAACAAT CTATGACCGA ATTCGACGAC ATCAAAAACC 7800

FIGURE 3-11

TCTCTTTACC TGAAACCCGT GACGCGGCGA AGCAGCTCCT CGACAGTGTC GCCGTGTGAC 7860

CTGACCGGTG AGGCGGCGCA GCGTTATTCA GGCGCTGACG CGCCACGCCG AGGAACTGCG 7920

GGCGGAGCAG CGCCGCCGCG GCCGCGAAGC CGAGGAGGAG CTGCGCCGCT ACCGGGCCGG 7980

TGAGCTGAGG GTGGTGCCCG GCGCTCCCAC CGGCGGCGAC GACGGCGACG CGCCGCCGGG 8040

CAACTCGTTG CGGGACACCG CGTTTCGCAC ACTGGATTCT TGTGTGCGAG ACGGCCTGAT 8100

GTCGTCGCGG GCGGCGGAGA CCGCGGAAAC CTTGTGCCGC ACCGGGCCGC CGCAGTCCAC 8160

CTCGTGGGCG CAGCGCTGGC TGGCGGCCAC CGGCAGCCGC GACTATTTGG GCGCGTTCGT 8220

CAAGCGGGTT TCCAATCCTG TTGCGGGGCA CACGGTTTGG ACCGACCGGG AAGCGGCCGC 8280

GTGGCGTGAG GCTGCCGCGG TGGCCGCCGA GCAGCGAGCG ATGGGCCTGG TGGACACCCA 8340

AGGCGGGTTT CTGATCCCGG CGGCGCTGGA CCCGGCGATC CTGCTGTCGG GTGATGGGTC 8400

GACGAACCCG ATTCGGCAGG TGGCGAGGGT GGTGCAAACG ACCTCCGAGA TTTGGCGGGG 8460

CGTGACTTCC GAAGGCGCCG AAGCTCGTTG GTACTCCGAA GCCCAGGAGG TGTCCGACGA 8520

TTCGCCAGCG TTGGCCCAGC CGGCGGTGCC GAACTACCGT GGAAGCTGCT GGATTCCGTT 8580

FIGURE 3-12


```
CTCCATCGAG CTGGAGGGTG ACGCGGCGAG CTTCGTTGGC GAGATCGGCA AGATTCTCGC    8640

GGACAGCGTT GAGCAACTGC AGACCGCGGC GTTCGTCAAC GGCTCCGGCA ACGGCGAGCC    8700

CACCGGGTTC GTCAGCGCGC TAACCGGCAC CTCCGATCAG GTGGTCGTCG GCGCGGGGTC    8760

AGAAGCGATT GTGGCGGCGG ATGTTTACGC GTTGCAGTCG GCGCTGCCGC CAAGGTTCCA    8820

GGCCAGCGCC GCGTTCGCGG CGAACTTGTC CACCATCAAC ACGTTGCGGC AGGCGGAAAC    8880

TTCGAATGGC GCGCTGAAAT TCCCATCGCT GCACGACAGT CCGCCGATGC TAGCCGGGAA    8940

GTCTGTCCTG GAAGTCTCCC ACATGGACAC CGTTGATTCG GCGGTGACAG CGACGAATCA    9000

TCCACTGGTG CTTGGCGACT GGAAGCAATT CCTCATCGTC GACAGAGTTG GGTCCATGGT    9060

GGAGTTGGTG CCTCACCTGT TCGGGCCGAA TCGCCGGCCG ACCGGGCAGC GCGGATTCTT    9120

CGCCTGGTTC AGGGTCGGAT CAGATGTGCT GGTGCGCAAC GCGTTTCGAG TTCTGAAGGT    9180

GGAGACTACC GCGTAGGTAG GATAGGGCCA GGCGTGGGCG GCCTCTGCTT AGGGGTGCCG    9240

GGCCGGCCAC GCCCGCCAAC TCCCCTGCGG GTTGCGTTGT CGATTCGTNN NNNNNNNNNN    9300

NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN    9360
```

FIGURE 3-13


```
NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN    9420
NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN    9480
NNNNNNNNCC AAGCCAGAAT ATCGAGCCTG GCGGCCATGG TCGCCGCCTT CCTGTTGCCG    9540
CTGCTTGGCT TTCGGCCGTT CCAGCTCGGC GATCCGGCGG CCAGCGGCGC CATTTGTTTC    9600
TCCGCGAACA GGCGGATTTC TTTGTCGTCG CTGCGGGTTG CGTTGTCGAT TCGTTTGAGC    9660
CGCTTGTAGG TGCCGGCGGA GATGCCGAGG GCTGCGCCTA CCTCCTTGTC AGTGTGGCGC    9720
TGAGACGGCT TTGGTTCCAT GGGACCAAAG CCGGCATTGG TGATCGATGC ACCGAGGCGA    9780
CCACCCTCGC GTTGGCGCTC CTTGGCTTTC GGGCGTTCCA GCTCGGCGAT CCGGCGACCA    9840
GCGGCGCCAT TTGTTTCTCC GCGAACCGGC GGATTTCTTT GTCGTCGCTG TGGGTTGCGT    9900
TGTCGATTCG TTTGAGCCGC CGGTAGGTGC CGGCGGAGAT GCCGAGGGCT GCGCCGATAG    9960
CAGTGTCTGT TTTCGTCGAA TGACGCTCTG ATTCTGGTTT GTAGCCCATG GGCCCCAAAC   10020
CAGAATATCG AGCCTGGCGG CCATGATCCT GCCCCTCGCG CTGCCGCTGC TTGGCTTTCG   10080
GCCGCTCCAG CTCCGCGATC CGGCGGCCAG CGGCGCCATT TGTTTCTCCG AGATAGCTTC   10140
```

FIGURE 3-14


```
CGGCCCATGG GCCGGAAGCT ATCCATGCCC CGCCCGTGGG ACCGCCCAGC GTCCTGTTGC   10200
CGCGGTGTTC ACCGTCAGCG CTCGTCTTCC GCTGGGCTTC CGCCGCCAAG CCCGATCATC   10260
CGGCTCCACG GCGGGGTGTC GTCGCCGTCG GGCTCGTCGT CGCCGGCGAG TCCGAGTTGC   10320
CGGTTGATGG CGGCTTGTTC CGGCCCGGAG CGGCCGGCCA GGATCGCCGG GCCGCACTCG   10380
TCGCCGCCGG CGGCGTCGGC GTGCTCGACG CTGATCCGCA GGAACGCTTC GAGCTCGCCG   10440
GTGTGCTCGT GCCGATTCAA CGCGGCCTGC AGCCGAACGA GCGCTTCCCT CACCTCCAAG   10500
GGCGCGTCCG GGAACCACGC CCGGATCGTC TCGGCCACCT GGTCGCGGTC GCAGACGGCG   10560
CGGTCACCGG TTTCCAGGTC GGTTACCGTC ACCAAGTGGT TGAAACGTGC TGGTGTGGTG   10620
GTCATGGTTG ATCTCCTGGC GTGGAATGTT CTTCAGCAGT CCACGGCCAA CCCCGCACCA   10680
ACACCTTCCA CCACCACGAG AAGCTGCTAC GCCACAACGA CGAGGACAAC CACGACGATC   10740
CGTGAGAATC GCCGCCCGCG AAGATCTTTG GACATCCCCA CATCGACGTG CGTCCTCGCC   10800
ACCTGGCCAG CACCCGCCCG AACCCGCGAG CTGGCCATTA AGACGAAGTT GCGATCAAAC   10860
CCCTTCGCCA TCAAGCTTTT TGGGCCCGCC TCACCCCAGC AGGTACTCGG CACGCGTGTT   10920
```

FIGURE 3-15

GCCGTCCCAG CGGCGCAAGC CGGCGAACCG ACCGTCAATC CGCGACGGCC GTCCCGCAAT 10980

GCGCAGCGCC CGCCCCAATT GGTGACCACC GACCCGTCGG GCCAGGGTGA CATGGGCGGT 11040

CCACTGACCG GGCAGGCTGT TGGCCATCGG CGCGGGCGCC AGGTGCGGGC CGCAGAGCCG 11100

GTGCACCTCG GCATGCAGGG CCAAAAGCTC GCTGGTCGGC ACCACCAGCC GGGTGAACAC 11160

GACATTGGCC CGCCCGAACA GCACCGGCGC GCCGATCACG CAGTCCAGCG GCAGCCGACG 11220

GGCAACCGCA CCCAGCGGCT CATCGACCTC CGGGGCGATC CGTTCGGCCA CCGCCAGCGA 11280

CACGTGCGGA CGGCTGGCCG GCGCCTGGCT GGGTATGCCG GCGGCGGCCA ACCCCGCCCA 11340

GATGCGCCGG ATCGCCGCCT CGGTATCGCT GTCGAAGACC AGCTCGATCG AATGCACCAT 11400

CAGCCGACCA GCCCGGCAAC CCAGTTGCGG TCGAACGCCG CCGCGCTCAT CGCCGCGAAG 11460

TCCCCGGCAT CCAGCGACGC GGCCCCGGCG GGCAGAGCGG CCCGCACCGT AGCAATGCGC 11520

GCCAGCGCGG ACCGATTCGA GGCTGCCACC AACCCGGGCG GGTCCGGCCA GCTGCCGATC 11580

ACCAGCCCTG CACATGAAAC CTGTTGTGCA GCAAGCGCTT CCAACGTCAA CTTGGTGTGG 11640

TTGAGGGTGC CCAGGTCCGC GGTGACCACC ACCAAAGCCG CGGCGGCCAC GTCGACGGCG 11700

FIGURE 3-16

ACATCGCGCA GCGTGACGCC CGGCTCGGCG AGTTCGACCA GCAGCCCGCC CGCCCCCTCG 11760

ACGAGGGTCA ACCGCCCGGG ACGGTCCAGG TCTGCGATCA GCCGCACGAT CTGATCGCGG 11820

GCGGGCAACG CCATCCCGGC GTGTTCGGCG GCGGCGGCCG GGGCCATCGG CTGCGGATAT 11880

CGCGCCAAGC CGGCCAGCTG GGTCACCCCG GCCAACCGGC CGACCTCGGC GAGGTCGTCG 11940

TCACCGCGGG CGGTGCCGGT CTGAACGGGC TTGCACACCG CCACGTCGAT GCCGGCCTGA 12000

CGTGCGGCCG ACGCCAGCGC CGCGCAGACG ACCGTCTTGC CGACCCCCGT GCCGGTCCCG 12060

GTGACGACCA GGATCGTCAA CGGCGCGCCA CGGCGAGAAC ATCCGTCAGC ACCCGCCGGG 12120

CCAGCTCGAG CTCGCCGGCG TTCAGCGATG CGCGCGCGGT CAGCCGCAGC CGCGACGTAC 12180

CCGCGGGCAC CGTCGGCGGC CGGAAGCAGC CCACCTTGAC CCCGGCGTCC AGGCAGGCCG 12240

CCGCGGCGGC CACTGCCGAC TCCGGCTCGC CCAGGATCAC CGACACCATC GCCGAGTCCG 12300

GCACCGCAGC CACACCGCAC ATCCGCGCAA GTTCACCAGC GTGGTTGAGC ACCGCCTGCG 12360

ATCGCCACGG CTCGGCCTGC AAGACGCGCA GCGCGGCCCG TGCGGCACCT TC 12412

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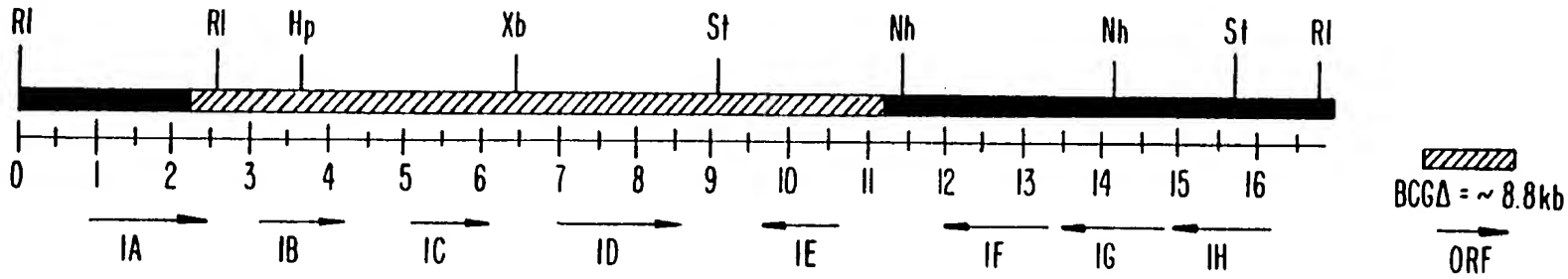


ORF	*M. tuberculosis* CODON USAGE	ORF SIZE (BASE PAIRS)	START - STOP (BASE PAIRS)	POSSIBLE RIBOSOME BINDING SITES	ENCODED PROTEIN (MAX.~kDa)	HOMOLOGIES TO PREDICTED ENCODED PROTEIN	P VALUE	HOMOLOGUE ACCESSION #
IA	YES	1542	889-2433	AGGA (10)	57			
IB	YES	1071	3130-4203 3139-4203	GGA (4) GGA(9)	36	*M. leprae* aceA BCG uraA	1.4e-14 3.0e-13	Z46257 U01072
IC	YES	969	5075-6046	GAGG(5)	34	*M. tuberculosis* esat6	2.3e-43	X79562
ID	YES	1657	6954-8612	NONE	59			
IE	YES	954	10619-9663	GGA(5)	34			
IF	YES	1380	13328-11946	AGGA(9)	48			
IG	YES	1386	14823-13438 14643-13438 14541-13438	NONE GGA(11) AGGAGA (10)	50			
IH	YES	1368	16190-14820	GAA (5)	46	*B. subtilis* subtilisin SERINE PROTEASES	3.6e-16	L29506

FIG. 4.

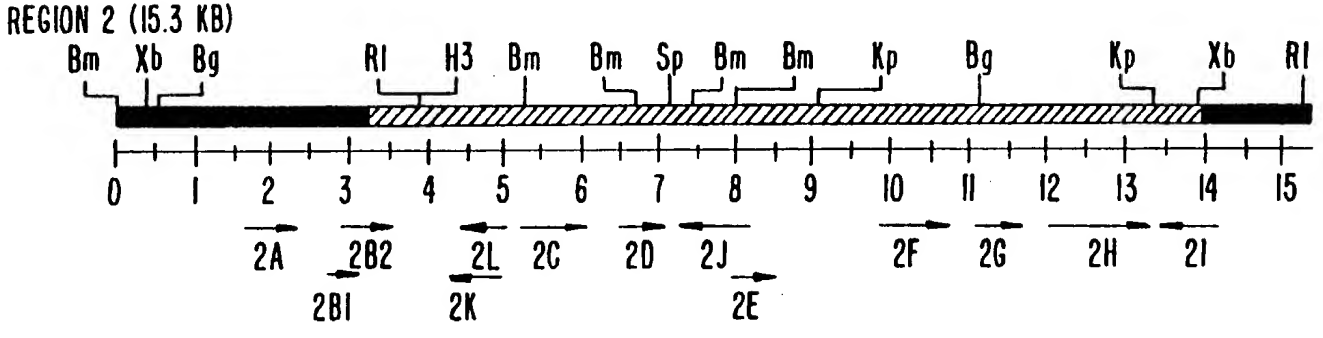


FIG. 5.

ORF	*M. tuberculosis* CODON USAGE	ORF SIZE (BASE PAIRS)	START - STOP (BASE PAIRS)	POSSIBLE RIBOSOME BINDING SITES	ENCODED PROTEIN (MAX.~kDa)	HOMOLOGIES TO PREDICTED ENCODED PROTEIN	P VALUE	HOMOLOGUE ACCESSION #
2A	YES	558	1829-2386	AGGGAG (7)	25			
2B1	YES	437	2862-3298	AGAA (4)	16			
2B2	YES	588	3003-3590	NONE	34			
2C	YES	948	5187-6134 5376-6134	AG? (8) GGA (8)	34	*E.coli* iciA lysR FAMILY	9.9e-47 <1e-5	P24194
2D	YES	657	6561-7217	NONE	22	*MLEPRAE* COSMID B1620 ORF CUTINASES	1.5e-7 ~4.e-5	U00015 A00975 U03393
2E	YES	522	8036-8560	NONE	19			
2F		966	9941-10909	AGGA (11)	37	*E.coli* iciA proUVWX	9.9e-146 2.7e-36	X73226 X17445
2G	YES	666	11118-11783	AAGA (6)	24	*M. tuberculosis* mpt64	6.7e-141	A30545
2H	YES	1443	11965-13407	AG (10)	51	*E.coli* gabP PERMEASE *Styphimurium* asp PERMEASE *T. harzianum* indal gene RETROVIRAL RECEPTOR	3.1e-11 1.4e-08 4.4e-11 2.5e-09	X65104 U04851 Z22594 X59155
2I	YES	846	14221-13376	GGAAGA (6)	31			
2J	YES	1050	8259-7211 7939-7211 7931-7211	GAG (10) GGAA (8) GGA (9)	35			
2K	YES	666	4992-4327	NONE	25			
2L	YES	597	5117-4521	AG (10)	21			

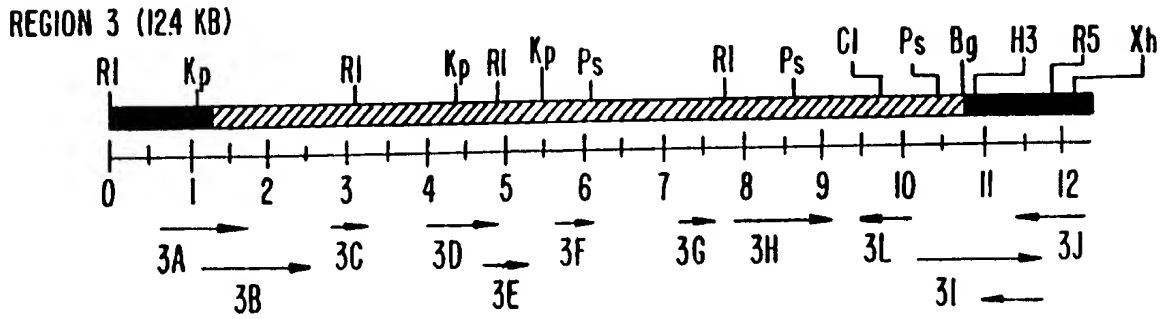
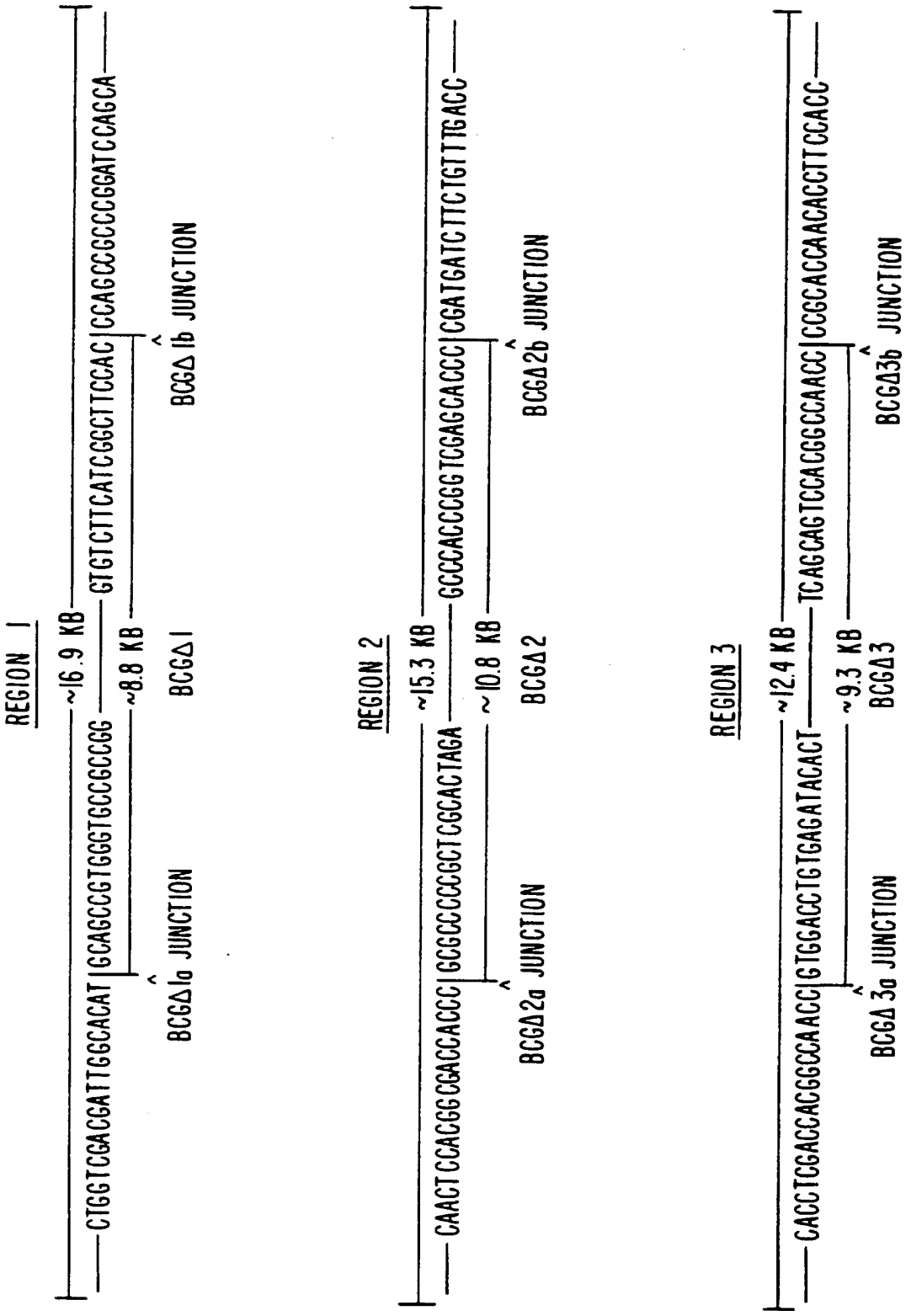


FIG. 6.

ORF	*M. tuberculosis* CODON USAGE	ORF SIZE (BASE PAIRS)	START - STOP (BASE PAIRS)	POSSIBLE RIBOSOME BINDING SITES	ENCODED PROTEIN (MAX.-kDa)	HOMOLOGIES TO PREDICTED ENCODED PROTEIN	P VALUE	HOMOLOGUE ACCESSION #
3A	YES	1143	613-1755	NONE	42	MTB mce sau3A *M. leprae* cosmid L247	2.9e-64 5.1e-13	X70901 U00021
3B	YES	1347	1214-2560		49	ACTINOPHAGE R4 attP GENE *B. subtil* SITE SPEC. RECOMB. RECOMBINASES/INVERTASES	3.0e-05 7.8e-4 8.2e-4	D90361 M29040 K00676 X01805, X07724
3C	YES	513	2820-3332	GGA (6)	19			
3D	NO NO	924	4007-4930 4070-4930	NONE GGA (7)	34	*S. coelicolor* phage phi-C31 EARLY REGION	4.2e-26	X76288
3E	NO YES	543	4795-5337 4915-5337	NONE GGAA(5)	21	pglY pglZ GENES	3.2e-11	X76288 L37531
3F	YES	576	5639-6214	GA(9)	20			
3G	YES	510	5253-7762 7285-7762	GAAGG(8)	19			
3H	YES	1330	7868-9197	GA(8)	47			
3I	YES	1665	10146-11810 10164-11810	GGAAG(6)	58	*M. leprae* B1170	6.2e-69	U00010
3J	YES	918	12319-11402	GAA (11)	30	*M. leprae* bioDAYB *C. glutamicum* bioD *B. sphaericus* bioDAYB	6.9e-53 2.6e-08 1.4e-05	U00010 D14083 M29292
3K	YES	702	11594-10893	AGG (4)	25	*M. leprae* cosmid B1170	1.0e-81	U00010
3L	YES	660	10147-??9488	AG(10)	25			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BCGΔ3b JUNCTION

FIG. 7.

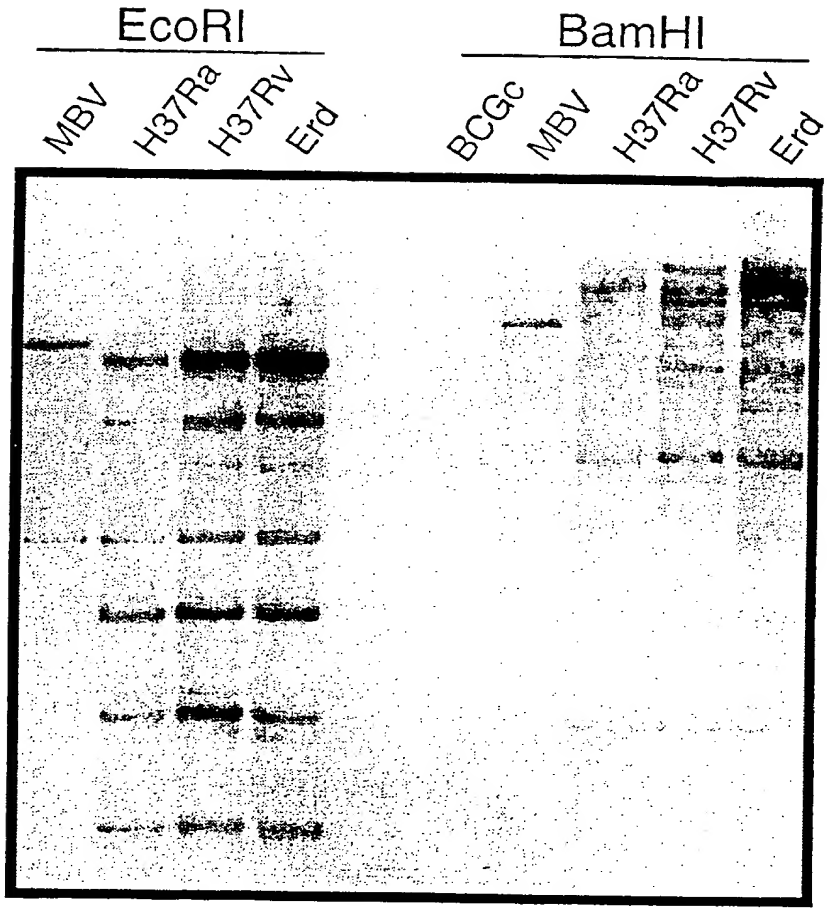

Figure 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01938

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 91.1, 91.2, 240.2, 252.3; 530/300, 350, 387.1, 388.1; 536/22.1, 23.1, 24.3, 24.32, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	Infection and Immunity, Volume 61, No. 5, issued May 1993, H. Li et al, "Evidence for absence of the MPB64 gene in some substrains of Mycobacterium bovis BCG", pages 1730-1734, see entire document.	1-10, 16, 17, 24, 25 ----- 18-23
X	JP, 1-247094 (AJINOMOTO ET AL) 02 October 1989, see entire document.	1-7
X	Infection and Immunity, Volume 59, No. 10, issued October 1991, C. Parra et al, "Isolation, characterization and molecular cloning of a specific mycobacterium tuberculosis antigen gene: identification of a species-specific sequence", pages 3411-3417, see entire document.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 APRIL 1996	Date of mailing of the international search report 29 MAY 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEFFREY FREDMAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01938

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Abstracts of the 1994 IDSA Annual Meeting,Clin. Infect. Dis., Volume 19, issued October 1994, R. Frothingham et al, "Sequence based strain differentiation in the Mycobacterium tuberculosis complex, including rapid identification of M. bovis BCG", page 565, see abstract 10.	1-25
X	R. GHERNA et al, "AMERICAN TYPE CULTURE COLLECTION: CATALOGUE OF BACTERIA AND PHAGES", Eighteenth edition, published 1992, pages 202 and 211, see entire document.	11-15
X ---- Y	Infection and Immunity, Volume 62, No. 4, issued April 1994, L. Pascopella et al, "Use of in vivo complementation in Mycobacterium tuberculosis to identify a genomic fragment associated with virulence", pages 1313-1319, see entire document.	1-7, 16-25 ---- 26
Y	Science, Volume 261, issued 10 September 1993, S. Arruda et al, "Cloning of an M. Tuberculosis DNA fragment associated with entry and survival inside cells", pages 1454-1457, see entire document.	1-23
X ---- Y	US,A,5,171,839 (PATARROYO) 15 December 1992, columns 5-10.	1-10 ---- 16-23
Y	Nature, Volume 256, issued 07 August 1975, C. Kohler et al, "Continuous cultures of fused cells secreting antibody of predefined specificity", pages 495-497, see entire document.	10
Y	US,A, 4,683,202 (MULLIS) 28 July 1987, see entire document.	16-22, 24, 25
Y	Genomics, Volume 4, issued 1989, D. Wu et al, "The ligation amplification reaction (LAR) amplification of specific DNA sequences using sequential rounds of template directed ligation", pages 560-569, see figure 2.	16-22, 24, 25
Y	US,A, 4,410,660 (STRAUS) 18 October 1983, columns 14 and 15.	23
Y	Gene, Volume 131, issued 1993, A. Kinger et al, "Identification and cloning of genes differentially expressed in the virulent strain of mycobacterium tuberculosis", pages 113-117, see page 114, column 2.	1-26
X,P	WO,A2,95/17511 (JACOBS ET AL) 29 June 1995, see entire document.	1-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01938

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,E	J. Bacteriol., Volume 178, No. 5, issued March 1996, G. Mahairas et al, "Molecular analysis of genetic differences between mycobacterium bovis BCG and virulent M. bovis", pages 1274-1282, see entire document.	1-26
Y, P	Microbiology, Volume 141, issued 1995, J. Rodriguez et al, "Species-specific identification of mycobacterium bovis by PCR", pages 2131-2138, see entire document.	1-7, 16-22, 24, 25
X ---- Y	Hybridoma, Volume 13, No. 1, issued 1994, A. Arya et al, "Production and characterization of new murine monoclonal antibodies reactive to mycobacterium tuberculosis", pages 21-30, see page 27, table 1.	8-10 ---- 16-23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01938

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12Q 1/68; G01N 33/53; C12P 19/34; C12N 5/10, 1/21; C07K 5/00, 14/00, 16/00; C07H 21/02, 21/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.1, 91.1, 91.2, 240.2, 252.3; 530/300, 350, 387.1, 388.1; 536/22.1, 23.1, 24.3, 24.32, 24.33

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, CAPLUS, WPIDS
search terms: mycobacter?, tubercul?, bovis?, BCG, calmette, guerin, DNA, RNA, oligo, nucleic, oligonucleotide, hybridi?, probe, primer, amplif?, PCR, polymerase chain, ligase chain, LCR, attenuat?, immunoassay, antibod?, monoclon?, polyclon?, protein, peptide,
antigen, virulenc?, infect?

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DT   29-JUN-1998 (Rel. 56, Last updated, Version 1)
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DE   Mycobacterium tuberculosis H37Rv esat6 promoter region, L45 antigen
DE   homologous protein LHP (lhp) gene, complete cds, and early secreted
DE   antigenic target 6 kDa (esat6) gene, partial cds.
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KW   .
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OS   Mycobacterium tuberculosis
OC   Eubacteria; Firmicutes; Actinomycetes; Mycobacteria; Mycobacteriaceae;
OC   Mycobacterium.
XX
RN   [1]
RP   1-1069
RA   Berthet F.-X., Birk Rasmussen P., Andersen P., Gicquel B.;

RT   "Promoter analysis of the M. tuberculosis orf1C gene encoding the early
RT   secreted antigenic target 6 kDa (ESAT-6)";
RL   Unpublished.
XX
RN   [2]
RP   1-1069
RA   Berthet F.-X., Birk Rasmussen P., Andersen P., Gicquel B.;
RT   ;
RL   Submitted (19-MAY-1997) to the EMBL/GenBank/DDBJ databases.
RL   Mycobacterial Genetics Unit, Institut Pasteur, 25, rue de Dr Roux,
RL   Paris, 75 75724, France
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(i) APPLICANTS:

(A) NAME: INSTITUT PASTEUR

(A) NAME: STATENS SERUM INSTITUT

(ii) TITLE OF INVENTION: A POLYNUCLEOTIDE FUNCTIONALLY CODING FOR THE LHP PROTEIN FROM MYCOBACTERIUM TUBERCULOSIS, ITS BIOLOGICALLY ACTIVE DERIVATIVE FRAGMENTS, AS WELL AS

to: R55u027.Af004671 check: 4782 from: 1 to: 1069

ID AF004671 standard; DNA; PRO; 1069 BP.

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AC AF004671;
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NI g3253155
XX . . .

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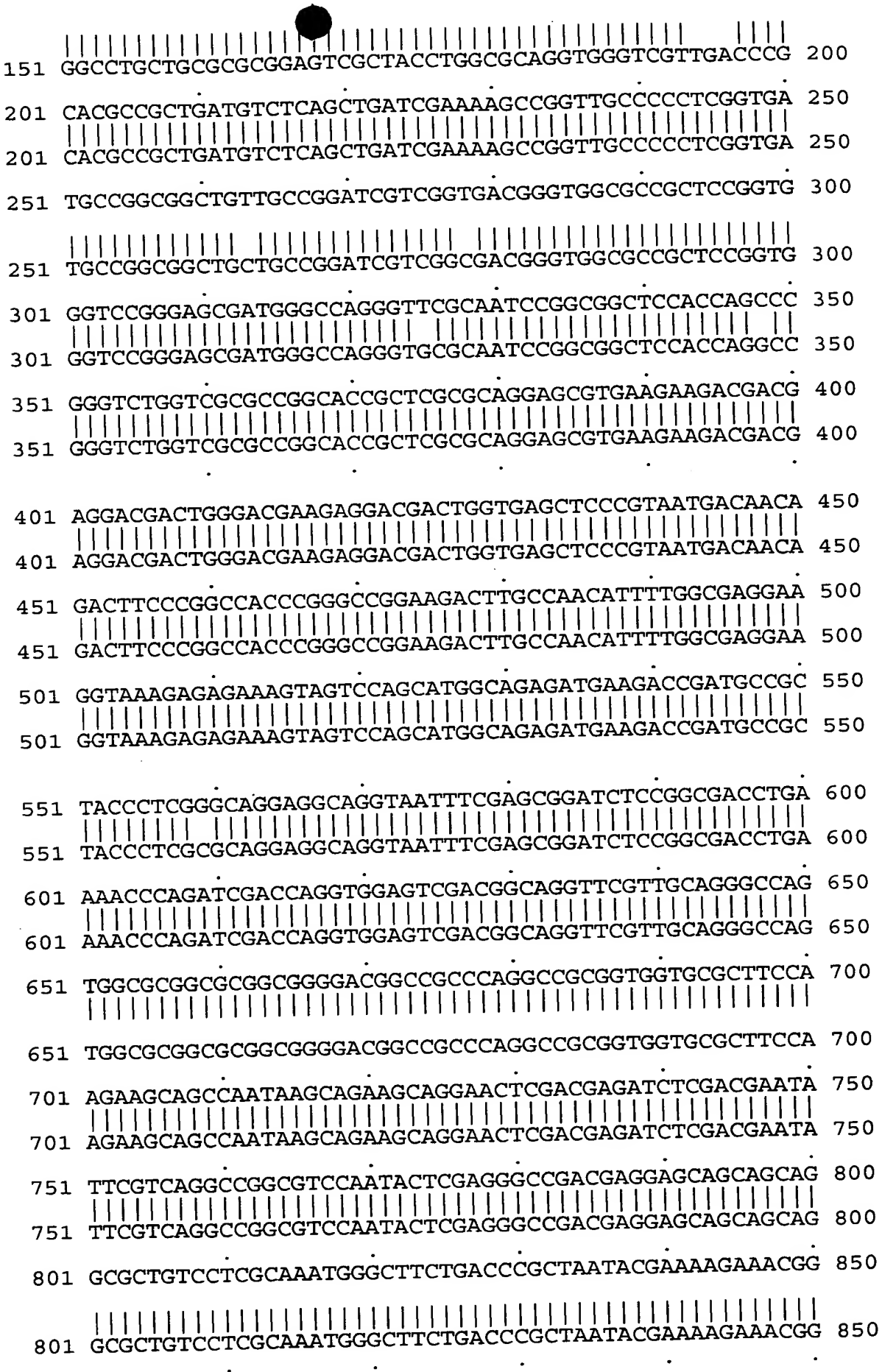

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